IPATIMUP
Instituto de Patologia e Imunologia Molecular da Universidade do Porto

Establishing the European Standard Set of forensic genetic markers in routine casework: population studies and next generation kit internal validation

Andreia Filipa Cabral de Melo
Forensic Genetics
Biology
2012

Supervisor
Cíntia Alves, Head of the Genetic Identification and Parentage Testing Unit, Institute of Molecular Pathology and Immunology of the University of Porto
Co-supervisor
António Amorim, Senior Professor, Faculty of Sciences, University of Porto

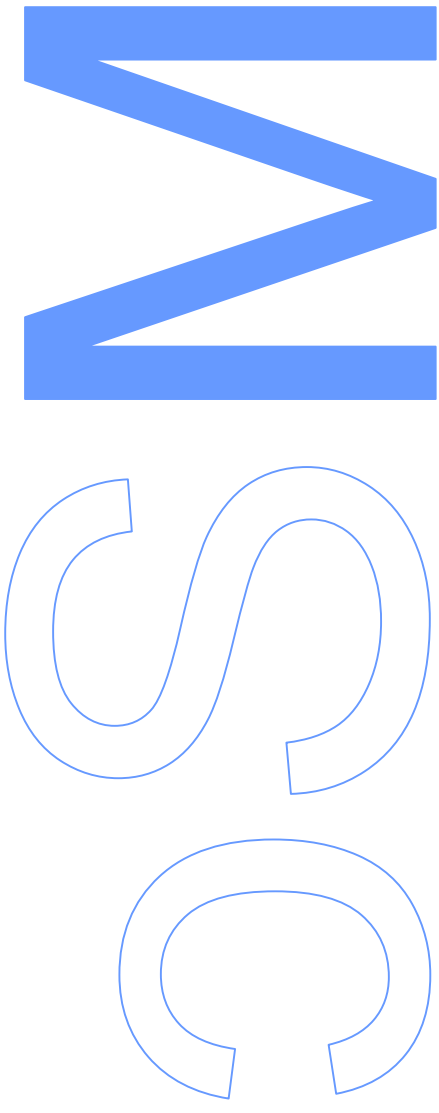
U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



Dissertação de candidatura ao grau de Mestre em Genética Forense apresentada à Faculdade de Ciências da Universidade do Porto

O presente trabalho foi desenvolvido sob a orientação científica da Drª. Cíntia Alves e do Professor Doutor António Amorim e realizado no IPATIMUP

Thesis presented for the degree of Master in Forensic Genetics, at the Faculty of Sciences, University of Porto

The present work was developed under the scientific supervision of Dr. Cíntia Alves and Professor António Amorim at IPATIMUP

AGRADECIMENTOS

À Cíntia, que ao longo de todo este caminho me guiou, orientou, me deu bases e conhecimentos para que eu pudesse realizar o meu trabalho, que esteve sempre lá, a qualquer hora, em qualquer dia… um grande obrigada por toda a confiança, compreensão e dedicação neste período! Obrigada pelo apoio, pela palavra amiga e entusiasmo no momento certo.

Ao Professor Amorim, que não só me permitiu estar presente neste Mestrado, como também me proporcionou a possibilidade de poder dedicar-me à área forense, no decorrer deste projeto. Um muito obrigada pela disponibilidade e pela opinião e palavra certa que me permitiu muitas vezes reajustar o meu pensamento crítico em relação a alguns assuntos.

Ao IPATIMUP, onde realizei todo o meu trabalho e onde me foi permitido conhecer e experimentar técnicas, procedimentos e análises forenses sem as quais o meu trabalho não faria qualquer sentido.

Agradeço ao grupo de Genética Populacional, nomeadamente às minhas "amigas de Mestrado" sem as quais este percurso não teria tido a mesma beleza. À Sofia, à Marisa, à Catarina X., à Ana, à Lídia e às duas meninas de Aveiro, Inês e Catarina S. um muito obrigada com muita amizade, pois tornaram este caminho inesquecível.

Aos meus pais, em especial à minha querida mãe, minha melhor amiga, que sempre me apoiou em qualquer passo ou caminho do meu percurso, sem nunca duvidar do meu talento. Espero orgulhar-te para sempre. À minha pequenina, minha maninha, obrigada pelo teu sorriso quando o dia não tinha corrido pelo melhor. Obrigada à minha linda família que mesmo longe me apoia e acarinha.

À Rosa e ao Joaquim, que fazem parte da minha família e que sempre me acarinharam.

Por fim, ao meu amigo, companheiro de sempre e para sempre, à minha metade, agradeço-te toda a compreensão, carinho e apoio incondicional. Sem ti não poderia ter chegado até aqui, pois seja qual for o desafio, iremos sempre ultrapassá-lo…juntos!

SUMMARY

National DNA profile databases are becoming the most powerful tool in forensic investigation and genetic identification. Overtime, European countries have been debating on standardization concerning the number and type of STR markers to be included, so that information exchange can be achieved in a more fruitful manner. The ESS (European Standard Set) core of loci was created consisting of five STR markers and this new set also accomplished the necessity to implement new markers which would be amplified in smaller amplicons, in order to solve increasingly common casework involving degraded DNA samples. This European request coincided with IPATIMUP's laboratory of genetic identification and kinship analysis necessity for more genetic information in order to obtain sounder conclusions in deficient paternities or in more complex kinship cases. Until now, the markers used on a routine basis in IPATIMUP´s laboratory are analysed through two multiplex systems, Identifiler Plus (Applied Biosystems) and Powerplex 16 HS (Promega), which amplify a total of 17 STRs and share 13 loci between them. However, when more genetic information was required, the laboratory accessed to an "in-house" multiplex, composed of 4 loci (CD4, F13A01, FES and MBPB), in order to obtain further autosomal information. These markers not only lack high polymorphism content but are in disuse by the scientific community. Consequently, participation in proficiency testing with these markers will not yield consensus, and so external quality control will not be accomplished. Therefore, the need for a new set of STRs became demanding, which also would preferably be more informative than the aforementioned "in-house" loci.

The ESSplex Plus kit is a next generation kit developed by Qiagen Company that contains the five new loci specified in the recently expanded European Standard Set (ESS) together with the remaining markers usually used in routine casework. In this work an internal validation study of ESSplex Plus kit was performed with evaluation of critical parameters such as system sensitivity, precision, contamination and mixture analyses, and degradation studies. A population genetic study was initially performed in order to first validate this new set of markers in the Portuguese population. Each new marker (D1S1656, D2S441, D10S1248, D12S391 and D22S1045) was characterized in terms of allele frequency estimation, Hardy-Weinberg equilibrium test and parameters of forensic interest; as well as a segregation analysis. Moreover, another next generation system, NGM kit (Applied Biosystems), was also used for a

concordance study (ESSplex Plus and NGM share the same loci) and for performance comparison purposes.

This internal validation study is crucial for understanding the performance, limitations and potentials of this system in the Portuguese population, applied in a particular laboratory with specific routine casework and procedures. In our investigation, 370 individuals were sampled, comprising 120 true trios (125 fathers, 125 mothers and 120 sons/daughters) for population and segregation studies. Moreover, for the kit performance studies, nine technical and administrative personnel from IPATIMUP Diagnostics were also sampled for different biological material in distinct sampling devices. The methodology adopted and used was adapted from the available literature on internal validation cases and also from SWGDAM guidelines.

No deviations from Hardy-Weinberg equilibrium were detected for the five new loci in the Portuguese population. Comparison of our sample with other available European samples generally revealed no significant allele frequency differences.

No genotyping inconsistencies were observed between ESSplex Plus and NGM kits in the concordance study, as well as between all four multiplex systems concerning the shared markers.

Comparing with the other systems evaluated (NGM, Identifiler Plus and Powerplex 16 HS), ESSplex Plus kit revealed higher sensitivity and a better detection of degraded DNA information.

Our results confirm the multiplex robustness regarding PCR chemistry and the improved performance requested by the European forensic community for typing degraded samples so as to ensure data quality and sensitivity. The five new ESS markers are suitable for application in our laboratory's routine casework as well as the ESSplex Plus kit as an option to be introduced in future routine casework.

Key-words:

ESSplex Plus, Internal Validation, European Standard Set, STRs, Next generation kit, Population studies, Forensic genetics.

RESUMO

As bases de dados nacionais de perfis genéticos estão a tornar-se uma poderosa ferramenta, ao nível da investigação forense e de identificação genética. Os países Europeus têm debatido a uniformização no que respeita ao número e tipo de marcadores STR a serem adotados, para que a informação partilhada entre eles possa ser obtida mais facilmente. Foi criado o ESS (*European Standard Set*), constituído por cinco marcadores STR. Este novo conjunto trouxe também a possibilidade de implementar novos marcadores com amplicões de menor tamanho, de forma a resolver casos que envolvam amostras de DNA degradadas, cada vez mais comuns na rotina laboratorial. Esta solicitação Europeia coincidiu com a necessidade de mais informação genética por parte do laboratório de identificação genética e análise de parentescos do IPATIMUP, para que conclusões mais sólidas fossem obtidas em casos de investigação de paternidade deficientes ou em parentescos mais complexos. Até agora, os marcadores utilizados na rotina do laboratório do IPATIMUP eram analisados através de dois sistemas em multiplex, Identifiler Plus (Applied Biosystems) e Powerplex 16 HS (Promega), que amplificam um total de 17 STRs e partilham entre si 13 loci. No entanto, quando era necessária mais informação genética, o laboratório acedia a um multiplex "in-house", composto por 4 loci (CD4, F13A01, FES e MBPB), de forma a obter mais informação de cariz autossómico. Estes marcadores não só carecem de um elevado conteúdo polimórfico mas estão também em desuso pela comunidade científica. Consequentemente, a participação em testes de proficiência com estes mesmos STRs não iria gerar consenso, sendo que deste modo o controlo externo da qualidade não seria conseguido. Deste modo, a necessidade de um novo conjunto de STRs tornou-se essencial, sendo que este seria ainda mais informativo do que os mencionados loci "*in-house*".

O ESSplex Plus é um kit de nova geração desenvolvido pela companhia Qiagen, que contém os cinco novos loci especificados no recentemente expandido *European Standard Set* (ESS), juntamente com os restantes marcadores usualmente utilizados na casuística de rotina. Neste trabalho, foi realizado um estudo de validação interna do kit ESSplex Plus, com a avaliação de parâmetros críticos como a análise da sensibilidade, precisão, contaminação e mistura, bem como estudo de degradação. Foi inicialmente realizado um estudo de genética populacional de forma a validar

primeiramente este novo set de marcadores na população Portuguesa. Cada um dos novos marcadores (D1S1656, D2S441, D10S1248, D12S391 e D22S1045) foi caraterizado no que diz respeito à estimativa de frequências alélicas, equilíbrio de Hardy-Weinberg, parâmetros de interesse forense, bem como análise de segregação. Além disso, foi utilizado outro sistema de nova geração, o kit NGM (Applied Biosystems), para o estudo de concordância (ESSplex Plus e NGM partilham os mesmos loci) e para comparação de performances.

Este estudo de validação interna é crucial para o entendimento da performance, limitações e potencial deste kit na população Portuguesa, aplicado a um laboratório particular com uma casuística de rotina e procedimentos específicos. Na nossa investigação, 370 indivíduos foram amostrados, compreendendo 120 trios verdadeiros (125 pais, 125 mães e 120 filhos/filhas) para estudos populacionais e de segregação. Além disso, para o estudo da avaliação de performance do kit, nove indivíduos, pertencentes ao pessoal técnico e administrativo do departamento de Diagnóstico do IPATIMUP, foram também amostrados, testando-se diferentes materiais biológicos em distintos suportes de amostragem. A metodologia adotada e utilizada foi adaptada da literatura disponível acerca de casos de validação interna, bem como das diretrizes de SWGDAM (*Scientific Working Group for DNA Analysis Methods*).

Não foram detetados quaisquer desvios ao equilíbrio de Hardy-Weinberg para os cinco novos loci, na população Portuguesa. Na comparação da nossa amostra populacional com outras amostras Europeias disponíveis, não foram detetadas diferenças significativas ao nível das frequências alélicas.

Nenhuma inconsistência genotípica foi observada entre os kits ESSplex Plus e NGM no estudo de concordância, tal como entre os quatro sistemas multiplex analisados no que diz respeito aos marcadores partilhados.

Comparativamente aos outros sistemas avaliados (NGM, Identifiler Plus e Powerplex 16 HS) o kit ESSplex Plus revelou uma maior sensibilidade bem como uma melhor capacidade de deteção de material genético degradado.

Os nossos resultados confirmam a robustez do multiplex no que respeita à química da reação de PCR, bem como um melhor desempenho, requerido pela comunidade forense Europeia para a tipagem de amostras degradadas de modo a assegurar a qualidade e sensibilidade dos dados. Os cinco novos marcadores ESS estão

adequados para aplicação na nossa casuística de rotina, tal como o kit ESSplex Plus como uma opção a ser introduzida na rotina laboratorial.

Palavras-chave:

ESSplex Plus, Validação Interna, European Standard Set, STRs, Kit de Nova Geração, Estudos populacionais, Genética Forense.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AT	Analytical Threshold
bp	base pair
CAP	College of American Pathologists
CODIS	Combined DNA Index System
CT	Threshold Cycle
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EDNAP	European DNA Profiling Group
EDTA	Ethylenediamine tetraacetic acid
ENFSI	European Network of Forensic Science Institutes
EPG	Electropherogram
ESS	European Standard Set
FBI	Federal Bureau of Investigation
FST	Fixation Index
FTA	Fast Technology for Analysis of Nucleic Acids
GHEP-ISFG	Spanish and Portuguese-Speaking Working Group of the ISFG
Indel	Insertion/Deletion
IPATIMUP	Institute of Molecular Pathology and Immunology of the University of Porto
ISFG	International Society for Forensic Genetics
ISFH	International Society for Forensic Haemogenetics

LINEs	Long Interspersed Elements
LTRs	Long Terminal Repeats
NTC	No Template Control
OL	Off-Ladder
PARF	Parentage/Relationship Proficiency Testing Exercises of paternity and forensic challenges by CAP
PCR	Polymerase Chain Reaction
PHR	Peak Height Ratio
PTC	Paternity Testing Commission
QA	Quality Assurance
QC	Quality Control
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Unit
RT-PCR	Real-Time Polymerase Chain Reaction
SD	Standard Deviation
SINEs	Short Interspersed Elements
SGM	Second Generation Multiplex
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
ST	Stochastic Threshold
STR	Short Tandem Repeat
SWGDAM	Scientific Working Group for DNA Analysis Methods
TWGDAM	Technical Working Group on DNA Analysis Methods
UV	Ultraviolet

1. INTRODUCTION

1.1. Short Tandem Repeat Markers and Commercial Multiplex Kits

1.1.1. Short Tandem Repeat Markers

The largest portion of the genome is composed of repetitive DNA. Part of this DNA is interspersed, with the repeat elements scattered throughout the genome. There are different types of repetitive elements, being the most common the short interspersed elements (SINEs), long interspersed elements (LINEs), long terminal repeats (LTRs) and DNA transposons. The other class of repetitive elements is tandemly repeated DNA. This can be separated into three different types: satellite DNA, minisatellites, and microsatellites (Goodwin *et al*., 2011).

The short tandem repeat (STR) DNA markers are microsatellites and belong to the class of length polymorphisms, being these repeat sequences usually located between genes, sometimes intronic and rarely exonic, varying in size from person to person (Butler, 2005).

These kind of markers differ in respect to the repeat structure, since they can be simple (Figure 1), compound, complex or even simple with non-consensus alleles (Figure 2). The simple repeats contain units that are identical in sequence and length, the compound ones contain two or more adjacent simple repeats varying in sequence and the complex repeats may present many repeat blocks of variable unit length or variable sequences. Furthermore the repeat motifs also differ by the length of the repeat unit: dinucleotide repeats are composed of two nucleotides tandemly repeated, trinucleotides by three, tetranucleotides by four and so on up to hexanucleotides which have six nucleotides in the core motif (Butler, 2005).

STR markers, introduced into casework in the mid-1990s, are the most commonly analysed genetic polymorphisms in forensic genetics (Goodwin *et al*., 2011), in terms of genetic identification and kinship analysis (Butler, 2005). The following properties justify this special categorization as forensic markers:

- Easy and fast amplification by PCR (usually < 400 bp);

- High polymorphism and discrimination power;
- Easy detection;
- Simultaneous analysis of several STRs, in a single test (multiplex), in automatic platforms with fluorescence detection;
- Multiplex commercial kits available;

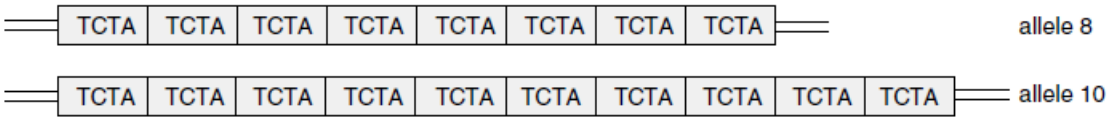


Figure 1 - Structure of a short tandem repeat marker. The core repeat varies between 1 and 6 bp (mononucleotide to hexanucleotide) being represented a simple tetranucleotide repeat. The alleles are named according to the number of repeats that they contain (Bär W. *et al*., 1997).

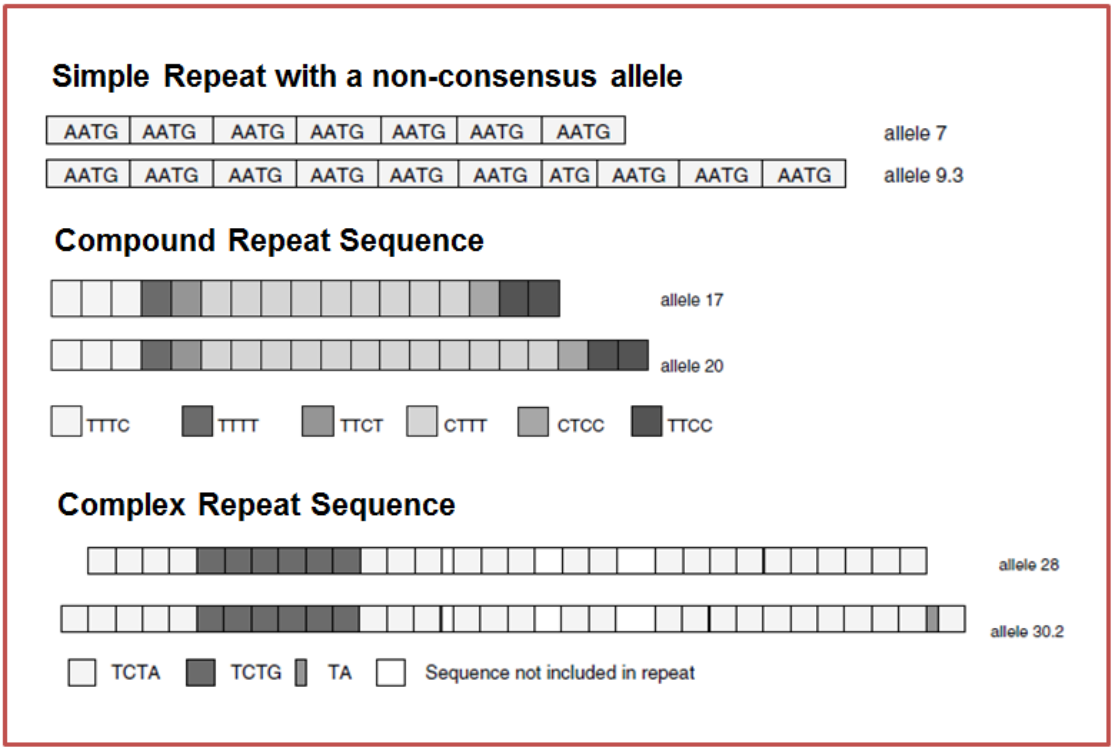


Figure 2 - Structure of different types of short tandem repeat markers. In the simple repeat with a non -consensus allele (microvariant), in the example, the 9.3 allele is missing an A from the seventh repeat. The compound repeat is composed by several elements. Finally the complex repeat sequence is also represented with non-consensus alleles that differ in both size and sequence (Butler, 2005).

There are also STR alleles which may contain some form of sequence variation, compared to more commonly observed alleles. They are often called microvariants because they have only a slightly different structure from full repeat alleles. Associated with this term, usually comes another one, off-ladder (OL) alleles, that refers to alleles that often do not size the same as consensus alleles present in the allelic ladder used as reference for genotyping samples (Butler, 2005).

Nowadays, the polymerase chain reaction (PCR) has been employed with unique flanking sequence primers to amplify DNA fragments containing STR loci (Sprecher *et al*., 1996). After the amplification process, the length of the products must be measured precisely. There was a limitation concerning the number of loci incorporated into the multiplexes, due to the allelic size ranges of the different loci, where overlap was not allowed. To overcome this fact, fluorescence labelling of PCR products followed by multicolour detection has been adopted by the forensic community (Lins *et al*., 1996, Sprecher *et al*., 1996). Thus, a series of fluorescent dyes has been developed and can be covalently attached to the 5' end of one of the PCR primers in each primer pair. Therefore, up to five different dyes can be used in a single analysis which allows for considerable overlap of loci (Buel *et al*., 1998, Ziegle *et al*., 1992). After analysing the raw data with the software, the end result is an electropherogram with a series of peaks that represent different alleles: the size, peak height and peak area are also measured.

During polymerase chain reaction (PCR) amplification of STR alleles, there are particular artefacts that can arise and interfere in the correct interpretation and genotyping of the alleles present in the DNA template. These stochastic effects may give rise to additional peaks besides the true alleles (Butler, 2005).

In the forensic genetics field, short tandem repeat markers with four or five base-pair core-repeat motifs are widely used and the most adopted by the forensic community (Bakker *et al*., 2005). This results from the fact that each STR locus has a tendency to generate a stutter product. Stutter is a phenomenon which derives from DNA polymerase slippage during PCR (Figure 3) and is dependent on the structure of the core repeats: shorter di- and trinucleotide repeats are more prone to stutter than are tetra and pentanucleotide repeats (Figure 3) (Bakker *et al*., 2005).

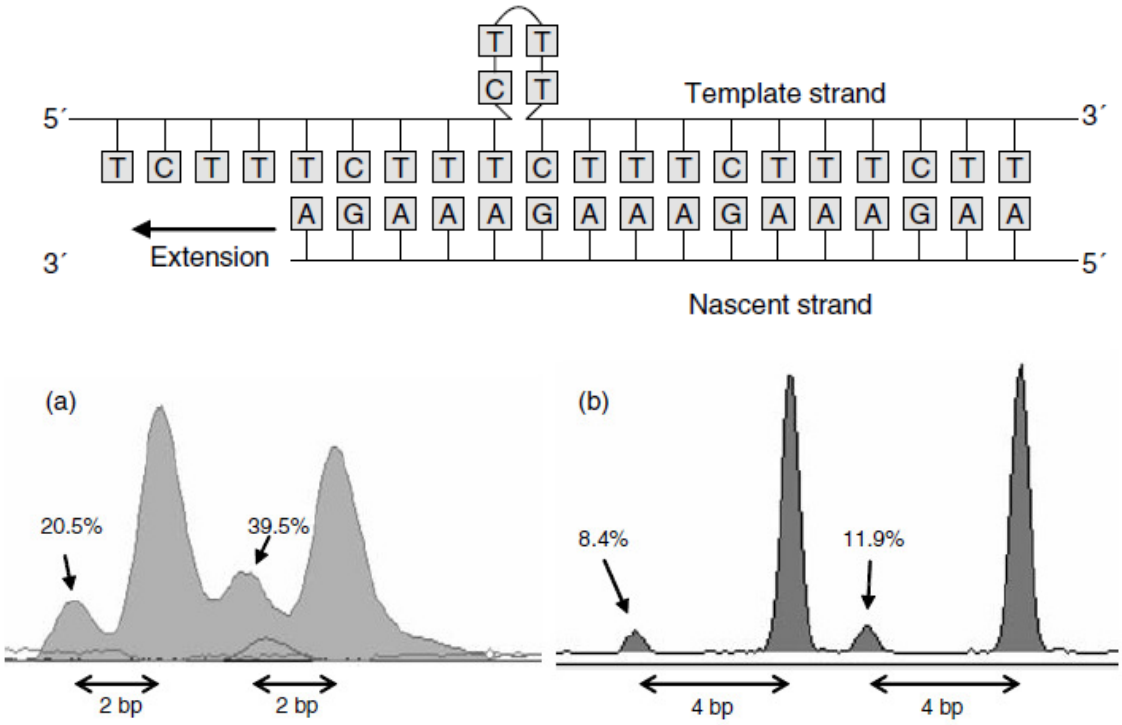


Figure 3 - The picture above shows the slippage process between the template and the nascent DNA strands. The copied strand represents the most common case and contains one repeat less than the template strand (n-1). The stutter peaks are indicated by the arrow and their size relative to the main peak is shown (based on peak area): (a) Dinucleotide repeat, which is prone to high levels of slippage. (b) Tetranucleotide repeat, which displays lower levels of stutter (Goodwin *et al.*, 2011).

The stutter effect always occurs and reflects the presence of smaller or larger peaks than each STR allele, and corresponds to a size difference related to the repeat motif. Even if stutters like n-2 and n+1 repeats (among others) may be observed, the most common stutter products are one repeat less (n-1) than the corresponding main allele. Measuring the percentage of stutter for each locus, may serve as a guideline for the detection of mixture samples. In a broad manner, if stutter ratios are introduced in the analysis software, this will act as a threshold for calling or not a stutter peak as a true allele for a certain locus. Nevertheless there are some parameters that can influence the stutter effect, such as the length and sequence inherent to the repeat motif (Applied Biosystems, 2011, Qiagen, 2010).

Another PCR derived artefact is the addition of an extra nucleotide to the 3′-end of a PCR product by the DNA polymerases, namely an adenine. This addition is a normal event during the copying process of the template strand. When this non-template addition occurs it results in a PCR product that is one base pair longer than the actual target sequence. If the process is incomplete, then a PCR product with fragment sizes

differing by one bp will co-exist, named split peaks (+/–A) (Figure 4). This process is referred to as 'adenylation' or the '+A' form of the amplicon (Clark, 1988).

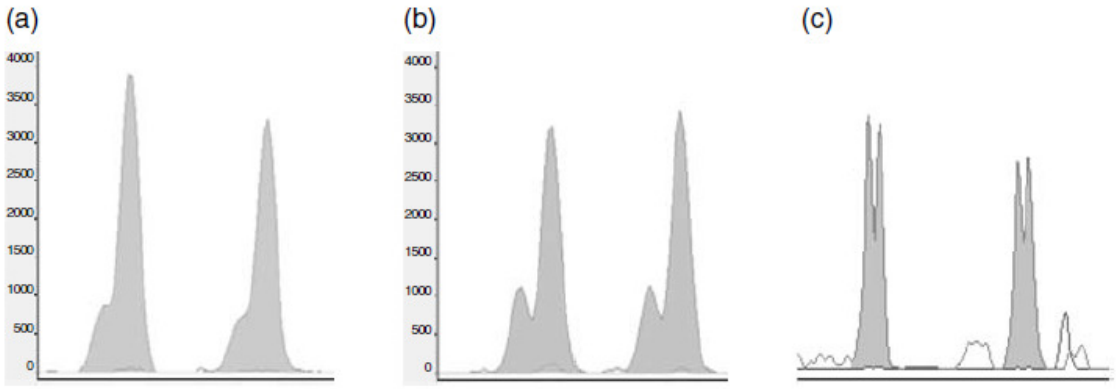


Figure 4 - Split peaks are seen in profiles when the non-template addition does not occur with all of the PCR products. The three examples show decreasing amounts of non-template addition with panel (a) showing an example where the vast majority of PCR product has the non-template addition through to panel (c), where only about 50% of the PCR product has the non-template addition (Goodwin *et al.*, 2011).

Stochastic events may also be observed when analysing STR markers. Allele drop-out is a process consisting in a failure to amplify, and therefore failure to detect an allele that exists in the template DNA. It is known that sequence polymorphisms can occur within or around STR repeat regions and that these variations can occur in three locations (relative to the primer binding sites): within the repeat region, in the flanking region, or in the primer-binding region (Figure 5) (Butler, 2005). Even if flanking sequences, around STR repeats, are fairly stable and consistent between samples, a polymorphism in these regions can result in silent alleles as consequence of primer hybridization problems (Kline *et al.*, 2011).

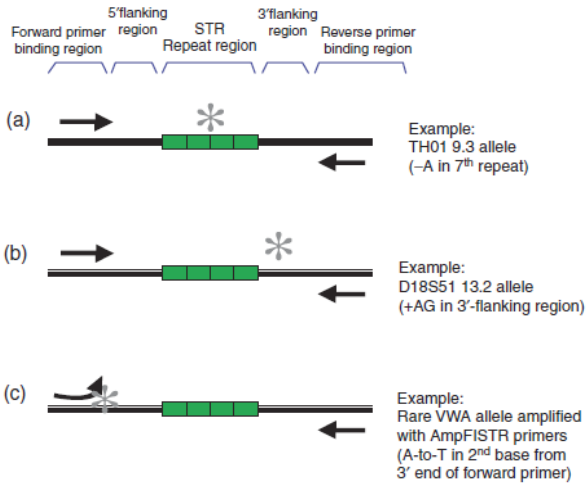


Figure 5 - (a) The variation occurs within the repeat region and should have no impact on the primer binding and the subsequent PCR amplification. (b) The sequence variation occurs just outside the repeat in the flanking region but interior to the primer annealing sites. Again, PCR should not be affected although the size of the PCR product may vary slightly. (c) The PCR can fail due to a disruption in the annealing of a primer because the primer no longer perfectly matches the DNA template sequence (Butler, 2005).

The drop-out event is also associated to low template DNA analysis, particularly of the larger STR loci (Figure 6), and degraded DNA, chemically modified (Goodwin *et al.*, 2011).

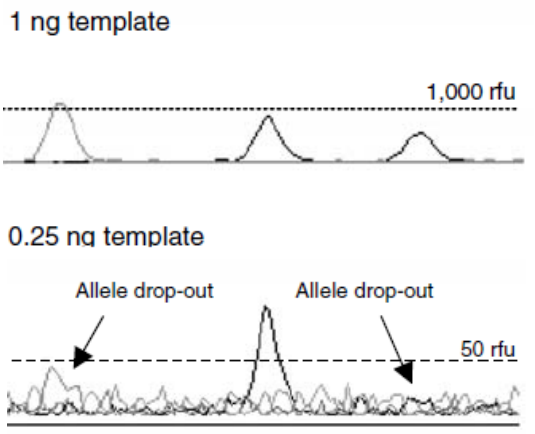


Figure 6 - With 1ng of template DNA, the peaks are well balanced and easy to interpret. When the PCR does not have enough template to amplify (0.25 ng), then allelic drop-out can occur (Goodwin *et al.*, 2011).

Allelic drop-in phenomena may also occur. It is mainly observed in low template DNA analysis because of contamination from environmental DNA. The main problem concerning this effect is related with a difficult interpretation of correct true alleles in a degraded or low copy number sample, since the appearance of extra peaks is inherent of this phenomenon (Schneider, 2007).

Another fact concerning STR loci analysis is related to equilibrium of alleles in heterozygous state. Two peaks in a STR profile are expected to be balanced in terms of peak height and area. However, peak height imbalance is very usual and variations in peak height can be due to chance events, where one allele is more efficiently amplified than another, for example due to a polymorphism in the annealing zone or to the presence of mixtures (real mixtures or contamination cases). Normally, an evaluation of the magnitude of heterozygous peaks is made, in order to achieve a threshold (peak height ratio), usually around 70% (Gilder *et al*., 2011), which may serve as a guideline for the detection of mixtures or drop-out.

There is also another phenomenon called pull-up that can emerge from the STR loci analysis. Since the dyes used to label amplified DNA fragments are at different wavelengths, there is some overlap in the emission spectra of these dyes. If an overamplified sample is analysed, this can saturate the matrix and results in a pull-up peak of another colour under the main peak. These pull-up peaks need to be differentiated from true alleles in order to obtain a correct interpretation, since that other colour may also be interpreted as an allele of another locus within the same multiplex (Clayton *et al*., 1998).

In order to be correctly applied in forensic genetic analysis, STR markers should be selected and studied taking into account specific properties, such as artefacts and stochastic effects, mutation rates, robustness in PCR, among others. It is also necessary to perform a population genetic study, in order to evaluate the behaviour of these markers in a particular population sample and their informativeness for forensic genetic applications.

1.1.2. Commercial Multiplex Kits

For the evolution and standardization of forensic genetics and for effectiveness of STR markers across a wide number of jurisdictions, a pressure was created to select a common set of standardized markers. The STR loci that are mostly used nowadays, were initially developed in the Dr. Thomas Caskey laboratory's, at the Baylor College of Medicine. Afterwards, Promega Corporation began the commercialization of these "Caskey markers" and Applied Biosystems also developed some new STR markers.

The selection of genetic markers should respect some particular features, namely (Goodwin *et al.*, 2011):

- Be discrete and have distinguishable alleles;
- The locus amplification should be robust;
- Should have a high power of discrimination;
- Should have absence of genetic linkage with other loci being analysed;
- Enable low levels of artefact formation during the amplification process;
- Have the ability of being amplified as part of a multiplex PCR.

A commercially available multiplex kit for the forensic field is composed by a set of reagents (primers and buffers containing a DNA polymerase amongst other components), previously tested by the manufacturer, in what concerns composition, quantity and performance, allowing for assurance of reliable results. The first multiplex commercial kit was developed in 1994, by Promega Corporation, known as Triplex CTT STR Multiplex System and was able to amplify 3 STR markers: CSF1PO, TPOX, and TH01 (Budowle *et al.*, 1997).

After that, a "quadruplex" kit was developed in the UK (United Kingdom) by the Forensic Science Service (and commercialized by Applied Biosystems), considered the "first-generation multiplex" for forensic analysis. In this multiplex kit 4 STRs were amplified in the same reaction: TH01, FES/FPS, VWA and F13A1 (Kimpton *et al.*, 1994).

Later, a commercial multiplex kit, known as “second-generation multiplex” (SGM), was developed again by the Forensic Science Service (also commercialized by Applied Biosystems) and replaced the “quadruplex” system. This multiplex allowed the amplification of six polymorphic STRs: TH01, VWA, FGA, D8S1179, D18S51, and D21S11 (Goodwin *et al*., 2011). After this period, with the introduction of fluorescent detection platforms, the development of STR kits that allows robust multiplex amplification of eight or more loci has revolutionized the forensic DNA field. STR profiles are now obtained in just a few hours which is an enormous breakthrough when compared with previous methods (for example, RFLP: restriction fragment length polymorphism) (Butler, 2005). The AmpF/STR ® SGM Plus ®, developed by Applied Biosystems, rapidly replaced the SGM kit and was adopted by many other countries around the world as one of their standard multiplex kits (Cotton *et al*., 2000).

At this time, in addition to the STR loci, the sex-determining Amelogenin locus, located in the X and Y chromosomes, was also incorporated into the most commonly used commercial multiplex kits (Thangaraj *et al*., 2002). After this, the USA selected 13 STR markers to incorporate its database management system named CODIS (Combined DNA Index System) and at this time several multiplexes were developed to cover these selected markers: CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11. Thereby, two major companies developed different commercial kits, for example, the AmpFlSTR ® Identifiler (Applied Biosystems) and the PowerPlex ® 16 (Promega Corporation), which allow the co-amplification of these 13 STRs in a single reaction, along with the amelogenin sex-typing marker and two additional STR loci (Figure 7) (Collins *et al*., 2004, Krenke *et al*., 2002).

Since that time, both Applied Biosystems and Promega Corporation have developed several commercial kits that address the needs of the DNA typing community and cover a common set of STR loci with matching probabilities that exceed one in a billion, in a single amplification reaction with 1ng or less of DNA sample (Butler, 2005).

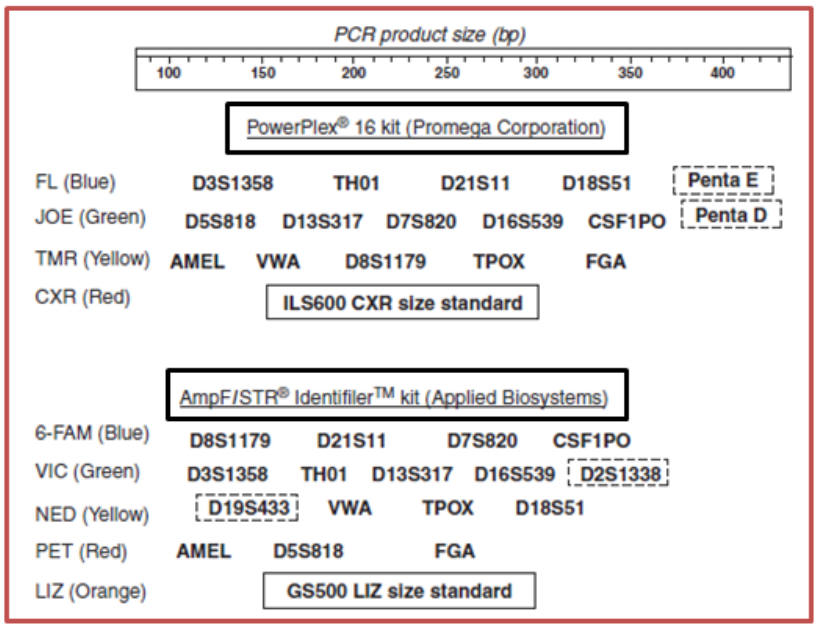


Figure 7 - Commercially available kits for the 13 CODIS core loci: Powerplex®16 and Identifiler (Butler, 2005). The genetic markers surrounded by black lines correspond to additional loci.

However, there are also laboratories that develop their own internal multiplexes. This kind of approach is time consuming and many resources are spent, which in many cases will not compensate the effort. Thus, the convenience of implementing these commercial kits is given by the fact that, on one hand, they are conveniently optimized and suffer criterious developmental validation and, on the other hand, allow opportunities for sharing data between laboratories since available kits determine which STRs will be used by the vast majority of forensic laboratories. This standardization allows the development of national DNA databases increasingly powerful in the forensic genetics field (Butler, 2005, Goodwin *et al*., 2011). Only very recently has a third multinational company started contributing in this field, Qiagen, who has proposed a new range of opportunities concerning multiplex forensic kits, trying to answer the need for more sensitive, robust, fast and accurate genotyping kits.

1.2. The new Standard Set of European Genetic Markers (ESS) in the forensic field and the Next Generation Kits

1.2.1. The new Standard Set of European Genetic Markers (ESS)

On the 25th of June of 2001, the Council of the European Union proposed the first list of genetic markers to be used in Europe by the Member States, for judicial purposes, respecting the main objectives of the European Union Treaty (February of 1992) and the resolution of this Council concerning the exchange of DNA results (June of 1997). Taking into account the growing importance of DNA in criminal investigation and the use of its effective exchange to oppose criminality, a list of 7 genetic markers was proposed (Table 1), composing the ESS (European Standard Set). The Member-States would have to adopt them and obtain DNA analysis results by using techniques previously validated, with these new loci. These kind of markers, for forensic purposes, must be devoid of any information about hereditary specific traits so to be authorized their use in the exchange of DNA information between member-states that adopt this new set (Union, 2001).

Table 1 - Composition of the first European Standard Set of Forensic Genetic Markers (ESS): D3S1358, VWA, D8S1179, D21S11, D18S51, TH01 and FGA.

The first ESS(European Standard Set)
D3S1358
VWA
D8S1179
D21S11
D18S51
TH01
FGA

Later, in July 2005, concerning terrorism related subjects, cross-border crime and illegal migration, some European countries such as Belgium, Germany, Spain, France, Luxembourg, Netherlands and Austria decided to take part in cross-border cooperation, in the well-known Convention of Prüm (Germany). In this way, the establishment of

national DNA analysis files had to be created by these members, for the investigation of criminal offences and the viable exchange of DNA information. This Convention determined the necessary procedures to generate automated searching and comparison of DNA profiles as well as issues concerning national contact points and technical information of the data (Union, 2005).

The Council of the European Union decided, in 2008, to implement a new decision concerning cross-border cooperation, particularly in combating terrorism and cross-border crime. The main aim of this decision refers to administrative and technical provisions, in particular the automated exchange of DNA data, dactyloscopic data and vehicle registration data. Regarding exchange of DNA-data, this decision comes to clarify several issues about this matter such as the recommendations for DNA database management, including criteria for addition or deletion of DNA profiles, matching rules and handling of partial profiles (Union, 2008).

Most recently, in 2009, on the exchange of DNA analysis results Council and regarding all work of the DNA Working Group of the European Network of Forensic Science Institutes (ENFSI), for the harmonization of the DNA markers and DNA technology across the years, it was proposed a new additional set of forensic genetic markers. Taking into account that an effective information exchange is facilitated by increasing the number of markers, and that the exchange of DNA data between Member States is rapidly progressing, it was necessary to expand the existing European Standard Set of loci (ESS) adopted in 2001 and provide more genetic information concerning loci that are capable of analysing degraded DNA samples. The European Standard Set (ESS) comprises nowadays the following DNA markers (Union, 2009):

Table 2 - Composition of the current European Standard Set of Forensic Genetic Markers (ESS): left column represent the first European Genetic Markers (2001) and in the right column is the new set of forensic genetic markers: D1S1656, D2S441, D10S1248, D12S391 and the D22S1045.

European Standard Set of Forensic Genetic Markers	
D3S1358	D1S1656
VWA	D2S441
D8S1179	D10S1248
D21S11	D12S391
D18S51	D22S1045
TH01	
FGA	

Once information was available relative to this new set of markers, members of the European Union were invited to use, at least, the DNA markers represented in Table 2, in order to facilitate an exchange of DNA analysis results. Thus, European members were invited to build up ESS analysis results in accordance with scientifically tested and approved DNA technology following the studies and frameworks developed by ENFSI.

It should be also remembered that these additional loci are not known to contain information about specific hereditary characteristics. If anything concerning this matter were to be discovered about a particular locus, member states were advised to no longer use that marker in DNA exchanges and also to delete any DNA results received (Union, 2009). The new set of five forensic genetic markers was initially characterized, concerning technical and specific parameters.

Table 3 - Locus – specific information of the new set of forensic genetic markers: D2S441, D10S1248, D22S1045, D1S1656 and D12S391 (Butler and Hill, 2012, Lareu *et al.*, 1998, Lareu *et al.*, 1996).

Locus	GenBank accession number	Repeat structure	Repeat motif	Lenght of the repeat unit	Chromosomal mapping
D2S441	AL079112	Simple	TCTA and TCAA [8-17] repeats	Tetranucleotide	2p14
D10S1248	AL391869	Simple	GGAA [7-19] repeats	Tetranucleotide	10q26.3
D22S1045	AL022314	Simple	ATT [7-20] repeats	Trinucleotide	22q12.3
D1S1656	NC_000001.9	Compound	TAGR [8-20.3] repeats	Tetranucleotide	1q42
D12S391	G08921	Compound	AGAT and AGAC [13-27.2] repeats	Tetranucleotide	12p13.2

In Table 3 are represented the markers that make part of the extended ESS (European Standard Set) and are contained in the main new Next Generation Kits directed for European countries: NGM™ and NGM SElect™ kits (Applied Biosystems), Powerplex®ESI and ESX Systems (Promega), and ESSplex SE and ESSplex Plus kits (Qiagen) (Butler and Hill, 2012).

1.2.2. The Next Generation Kits

Since national DNA databases are becoming the most powerful tool in forensic investigation and genetic identification, and at the same time each country was selecting the STR markers that allowed an inclusion in their own database, the ESS (European Standard Set) core of loci was created, trying to achieve the standardization of forensic genetics across Europe. However, the creation of this new set does not

mean that European countries should leave the loci that have been using in the construction of their national databases in favour of the new ones, but instead they would have to adopt and use these new markers in future analysis (Schneider, 2009).

Regarding this issue, ENFSI and the European DNA Profiling Group (EDNAP) have been working together to achieve standardization of DNA profiling throughout Europe, with the main purpose of facilitating the comparison of DNA profiles between laboratories (Gill *et al*., 2006a). The intention of these groups was the creation and design of multiplexes that could achieve the benefit of criminal justice systems, simultaneously increasing detection rates and reducing potential adventitious matches. This purpose is thought to be achieved by the adoption of this new set of genetic markers, increasing the chance of success in highly degraded samples and the discrimination power of all samples tested, since the decrease of amplicon sizes and the increase of the number of loci detected will be guaranteed (Gill *et al*., 2006b). In this way, the main goals for the new multiplexes were:

- Improve the discrimination power with the addition of more loci;
- Improve sensitivity of testing in order that smaller amounts of DNA may be detected;
- Improve robustness and quality through the more effective amplification of degraded DNA (Gill *et al*., 2006a).

Thus, from the results of collaborative experiments of the ENFSI/EDNAP groups, came a need to alter existing multiplexes to improve success rates when degraded DNA is analysed (Gill *et al*., 2006a). In fact, the EDNAP group demonstrated that low molecular weight STR loci show benefits in detecting degraded samples since they increase the chance of successful amplification by targeting smaller loci (Dixon *et al*., 2006). Damage to the DNA molecule can occur through exposure to environmental conditions, such as ultraviolet light, heat and humidity. Forensic samples are particularly prone to such damage due to their prolonged exposure after deposition (Diegoli *et al*., 2011). Unfortunately, amplicon size and the ability to amplify extremely degraded DNA molecules were not considered when the first STR loci were selected. Thus, several of them have a large number of repeats or wide allele ranges which are not appropriate for generating small amplicons. In this way, leaders of the European forensic DNA community recommended the addition of new mini-STR loci (capable of

small fragment amplification) to the 13 CODIS markers (Butler, 2006b). As a consequence of this study, a convergence of two initial strategies was proposed to produce a final product containing 15 loci: in strategy I, mini-STRs (D2S441, D22S1045 and D10S1248) were added to multiplexes that were already developed, providing a multiplex with 13 loci for countries using SGM plus kit. In a second strategy, a second group of loci (D12S391 and D1S1656) was preferred by some European countries, to be included in the new multiplexes, since these midi-STRs provide a higher discrimination power and their size was already reduced. Thus, the convergence of these two strategies intended to combine the advantages of strategy 1 and 2, resulting in new multiplexes composed, generally, by 15 loci plus Amelogenin. The choice for these particular 5 new genetic markers was due to the fact that they are located on separate chromosomes or are genetically unlinked from the widely used 13 CODIS markers (Gill *et al*., 2006b).

Regarding this new multiplex strategy, there was a need to develop commercially available kits with these ESS core markers. Concerning this need, the major commercial companies in the forensic field started several studies and developmental procedures in order to bring to the market the Next Generation Kits and a number of STR multiplexes fulfilling the ENFSI and EDNAP objectives. These autosomal kits have in common the fact that they share the same core of forensic markers (Figure 8). These 12 European core loci are typically accompanied by D16S539, D2S1338, D19S433 and SE33 markers (Schneider, 2009).

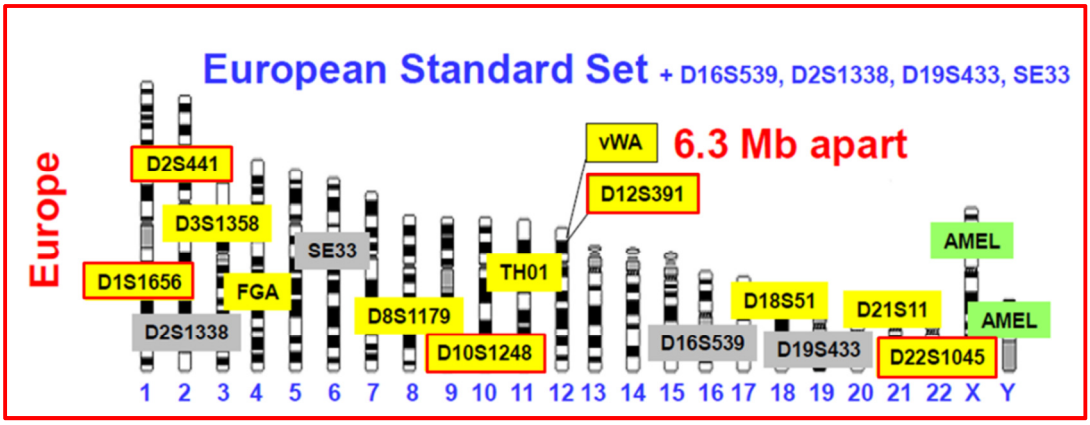


Figure 8 - Graphic representation of the European Standard Set core of genetic markers plus additional markers which frequently also composed the Next Generation Kits, shaded in grey (Butler, 2010b). The new set of genetic markers is surrounded with red lines.

Nowadays, the validated Next Generation Kits belonging to the three major companies are commercially available. However, if laboratories wish to adopt them they must first suffer vigorous internal validation, before their introduction in routine casework.

1.3. The Internal Validation Process: history, validation guidelines and studies

It was in the USA, in a few unaccredited laboratories, that some cases emerged where DNA analysis was compromised due to several problems, concerning building construction, techniques and laboratory personnel inadequately trained. The kind of complications encountered resulted later in wrongful decisions of the judicial system, illustrating the need for consistent internal quality assurance and external control to ensure accuracy within a laboratory (Butler, 2005). Thus, it was decided that any scientific procedure, test or product related with DNA typing which results in genetic information that may lead to the loss of liberty of any individual involved or not in a judicial process, needs to be implemented very carefully. Thereby, in order that this sort of process can be conducted properly and since DNA testing is always regarded as a powerful investigative tool for the law enforcement community, two main concepts were created concerning good laboratory practices and accurate scientific results: *Quality assurance* (QA) and *Quality control* (QC). QA is related to planned and systematic actions that are needed to ensure confidence in a product or service. On the other hand, QC is related to the routine operational techniques and the procedures used to fulfil requirements of quality.

Therefore, since these concepts are crucial in the development of forensic DNA technology, numerous organizations were established across the forensic community in order to recommend and inspect quality assurance guidelines and quality control measures (Butler, 2005).

1.3.1. Ensuring Quality: Guidelines and Accreditation

Validation guidelines for quality assurance programs, in DNA analysis, were first published in the USA, more exactly in 1989, when the Technical Working Group on DNA Analysis Methods (TWGDAM) was created, under FBI Laboratory sponsorship. These validation guidelines were later republished and updated in 1991 and 1995, and

in 1998 this organization changed its name to Scientific Working Group on DNA Analysis Methods (SWGDAM). Over the years, several subcommittees have elaborated numerous recommendations before the SWGDAM, concerning issues related to good practices in forensic genetics. In July 2004, SWGDAM published revised validation guidelines, being currently the most recent guidelines, and it operates nowadays as the responsible group for the recommendations concerning quality issues, to the forensic community within the USA (Butler, 2005, Butler *et al.*, 2004).

In Europe, the International Society for Forensic Genetics (ISFG), formerly International Society for Forensic Haemogenetics, ISFH), is an international organization responsible for the promotion of scientific knowledge in the genetic markers field, analysed for forensic purposes, and includes several members from 50 countries, with a strong impact in Europe. The ISFG has established a Paternity Testing Commission (PTC) which is concerned in establishing general standards for testing laboratories based on ISO 17025 standards (Morling *et al.*, 2002). However, it was in 1995 that the first European organization was created with the responsibility of commanding the set of standards for exchange of data, between European member states, namely ENFSI (Butler, 2005).

The European forensic DNA community has another organization, EDNAP, and is constituted by a dozen European nations. This organization is, in fact, a working group of the ISFG and has been conducting a series of inter-laboratory studies on STR markers, investigating the reproducibility of results from multiple laboratories, showing that with quality control measures excellent reproducibility can be obtained by these groups (Butler, 2005).

Nowadays, any forensic laboratory which performs DNA tests and analysis for other institutions, such as courts or private companies, among others, should undergo an accreditation process by an official organization. This process results from a successful audit by an accrediting body and requires that the laboratory demonstrates good lab practices regarding chain-of-custody and evidence handling procedures. A laboratory audit evaluates its entire operation and is conducted by its own laboratory staff (internal audit) or by an independent organization (external audit), taking into account pre-established guidelines. The results and records of each audit must be saved as a way to define what can be improved (Butler, 2005).

Proficiency tests are also mandatory and should be performed periodically. A proficiency test has the purpose of evaluating a laboratory´s performance in conducting DNA analysis procedures, by integrating the laboratory's normal routine work. Thus, these kind of tests also evaluate the ability to obtain a concordant result using the standard operating protocols (SOPs) approved for a certain laboratory. Regarding this issue, proficiency tests could be internal proficiency tests that are administered by a laboratory´s member or external proficiency tests that are administered by an external organization. If this sort of test is performed such that the laboratory staff does not know that a test is being made, then it is called a blind external proficiency test. This blind test is considered the most effective at monitoring a laboratory´s performance.

Although it can be rather expensive and time-consuming, the participation in proficiency – testing programs is a crucial part of a successful laboratory´s quality assurance. The main purposes of these tests are the standardization of methods and procedures, the standardization of nomenclature, the evaluation of the competence of a laboratory to obtain the correct result and the elimination of errors in typing (FBI, 2008).

In the accreditation process, one of the main issues is validation of methods. This is probably the most difficult and time-consuming task, but it must be undertaken before the introduction of any method in routine casework (Butler, 2005).

1.3.2. Validation

The validation process is an essential part of a quality assurance program in a laboratory, when new techniques and technologies are being introduced. This concept involves performing specific laboratory studies to verify if a new instrument, software program or product is working properly. In other words, a validation study is a process where a particular laboratory procedure is considered robust, reliable and reproducible. A robust method ensures successful results in a high percentage of the time while a reliable method refers to one where the obtained results are correct and reflect the samples being tested. Concerning a reproducible method, it represents the ability to obtain the same or similar results, each time a sample is tested. It is through the validation process that the scientific community acquires the necessary information about the conditions, limitations and specificities of a particular procedure (Butler, 2006a, Butler, 2007).

In the validation process there are two different levels that must be considered: developmental validation and internal validation. At the developmental validation level, the new STR loci or STR kits, new primer sets, new technologies and instruments are typically tested and evaluated by the manufacturer of a DNA testing company or by large laboratories. Moreover, when a laboratory develops their own methods, they should be submitted to developmental validation, such as the "in-house" PCR kits. On the other hand, the internal validation consists in verifying if the established procedures, previously examined by developmental validation, will work well in a particular laboratory. It is an in-house demonstration of the reliability and limitations of the procedure, conducted by each forensic DNA testing laboratory (SWGDAM, 2004).

The constant evolution of new methodologies in DNA testing, the advance of technologies and new DNA kits becoming available every day, promotes the need to validate and implement new procedures. In fact, if laboratories do not have an outlined validation plan, significant amount of time and money could be spent. In fact, the consequences of not performing a correct validation study can translate into loss of information, wasted resources and money in inappropriate volumes of reagents and lack of confidence by the judicial system in DNA proof results. Since reliable analytical data, concerning DNA results, is desirable in courts, validation can transmit confidence as well as aiding quality assurance in a particularly laboratory (Butler *et al*., 2004, Butler, 2007).

Therefore, in order to promote appropriate validation studies across European Forensic Laboratories, adequate standardization procedures should be defined. However, even with the great work that has been made in this direction, there is not yet a standardized validation strategy generally accepted or utilized across forensic DNA laboratories. There is a wide range of responses throughout the European community, concerning the examination of different validation studies, making it difficult to define specific recommendations. Nowadays, the most accepted and referenced procedure is the SWGDAM recommendations (Butler *et al*., 2004, Butler, 2007).

This great lack of standardization across European members, concerning validation parameters, allowed the spread of a number of misconceptions regarding validation (Butler, 2007). One of the wrong ideas resulting from this issue is precisely the thought that the validation process is uniformly performed throughout the DNA community. Auditors should understand that variability can always exist among validation studies since different perspectives exist. Perhaps as more validation studies and results are

completed and shared, a greater uniformity could be established concerning approaches, for conducting these studies throughout the human identity testing community (Butler *et al*., 2004, Butler, 2006a).

Another wrong idea is that the process of learning and training people, regarding a new technique, is part of the validation process. It is important to note that instruments are calibrated, people can be trained and methods are validated in order to verify if a particular process works adequately, when it is performed in a particular laboratory. Thus, the idea that hundreds or thousands of samples are required to validate an instrument or method is also wrong. The same reliable results and conclusions could be achieved with a fewer number of samples. SWGDAM has recommended that, when conducting an internal validation, a total of at least 50 samples should be analysed, not 50 samples per experiment, in order to control the validation process duration (Butler, 2006a).

Finally, regarding wrong ideas created around the validation concept, it should be reminded that this process does not mimic every situation but instead, it must be carefully planned in the different types of experiments that compose it and be used as internal additional information (Butler, 2006a).

1.3.2.1. Developmental Validation Studies

Regarding developmental validation of autosomal commercial kits, mostly performed by the respective company or corporation, the usual studies undertaken are indicated in bold and briefly described. The other studies mentioned thereafter are also usually part of the internal validation process.

- **Stability Study**

  This test is performed when there is a chance that the DNA analysis, deposited on various substrates, be potentially affected by different environmental and chemical conditions. In this case, known samples can be used to evaluate and determine these possible effects (SWGDAM, 2004).

- **Species Specificity**

In this study, the potential to detect DNA from forensically relevant nonhuman species, for techniques designed to type human DNA, is evaluated. Thus, the product that is under validation is studied in terms of the specificity for detecting just human alleles. Still, for techniques in which other than human species are expected to be targeted, the ability to detect non targeted species is also evaluated (SWGDAM, 2004).

- **Characterization of loci**

This test can be implemented when the issue under validation involves new genetic loci. In this case, topics as the nature of the polymorphism, their inheritance, their mapping in the respective chromosome and the evaluation of the event of genetic linkage are the most relevant in this analysis (SWGDAM, 2004).

- **Environmental study**

This study uses samples of known genotype and submits them to different environmental stresses, such as sunlight exposure, humidity and temperature fluctuations. It is supposed to evaluate and verify if the correct genotype is always obtained, in a sort of study which reflects the typical forensic cases (Butler, 2005, SWGDAM, 2004).

- **Annealing temperature studies**

This type of study usually tests a range of different temperatures, concerning the annealing process, using a specific PCR system and a genetic analyser to evaluate the data. The aim is to establish the optimum temperatures to obtain robust profiles and the temperatures where the performance of the reaction is compromised. This study is crucial because thermal cycler temperature is critical to assay performance (SWGDAM, 2004).

- **Cycle number studies**

  In this study, the number of PCR cycles needed to achieve the adequate amount of PCR product is evaluated in a range of different values around the optimum number of cycles previously determined. Although it is expected the increasing of PCR product amount with more cycles, this test has to take into account the nonspecific peaks that can be produced with this increase (SWGDAM, 2004).

- Population study
- Accuracy, precision and reproducibility studies
- Sensitivity study
- Stochastic effects study
- Mixture Study

1.3.2.2. Internal Validation Studies

When there is need for introducing a new commercial kit in a laboratory's routine, it has to be previously validated in order to understand its behaviour and limitations relatively to the space, instruments and techniques that will be used. The range of tests conducted for this purpose are not to be strictly followed but instead, they have to be seen as auxiliary internal guidelines that can be consulted when some doubt emerges in the routine casework.

The most representative studies that are usually suggested and recommended by international organizations are summarized in the following points:

Population Study

Populations have to be defined in a socio-anthropological context to be able to carry out research studies on allele frequency distributions. For example, if the genotype of a suspect matches the DNA profile of the evidence, there is still the possibility that this

match has occurred by chance. In kinship investigations this sort of study is equally relevant. Therefore, population studies are needed since a powerful DNA database is crucial, with correctly estimated allele frequencies and forensic parameters of interest, so that robust conclusions may be achieved (Schneider, 2007). To accomplish this task, as a guidance, the analysis of a population sample of about 200 unrelated individuals should be representative enough when using these type of markers (Applied Biosystems, 2011, Butler, 2005).

It is also important to confirm Mendelian inheritance of these markers. Autosomal STR alleles are known to be codominantly transmitted from parents to their children. Unless a mutation or other phenomena take place, it is expected that a child shares one allele from each parent. By testing a reasonable set of true trios (mother, father and son/daughter), transmission properties may be studied and phenomena such as mutation and silent alleles may be detected.

Concordance Study

This study consists in examining if a given sample, with a known profile, will have the same STR genotypes when using a different PCR amplification system, because it is expected that there are different sets of primers in the different PCR systems available, for each locus. In the evaluation of large sample numbers, variations can be expected in primer annealing regions which, in the worst case, can result in silent alleles. Thus, it is common to test for the same set of individuals from a population sample, different commercial multiplex kits with the same loci (Qiagen, 2010).

Sensitivity Study and Stochastic Effects

The sensitivity concept could be defined as the ability of a certain system to yield correct and reproducible results with a given input DNA. The point where the sensitivity of a system is insufficient to detect an allele, although it is present, is defined as the limit of detection. The importance of quantification is notorious when just with a dilution series of a known sample we can conceive the minimum quantity of input DNA needed to obtain a reliable result. Thus, with this study, not only cost-effectiveness may be achieved in the long run, since it is expected less need for replications, but also

guarantees that results may be reliably interpreted since a balanced profile should be always achieved.

However, with inadequate amounts of input DNA, stochastic effects can emerge. Thus, if too much DNA is added to the PCR reaction different types of phenomena can be observed, such as the “pull-up” effect, incomplete +A nucleotide addition and a higher stutter effect which may compromise the final interpretation. On the other hand, when a sample contains very low amounts of input DNA, effects such as unbalanced heterozygous alleles, “allelic drop-in” and “drop-out” may be observed (Applied Biosystems, 2011, Qiagen, 2010).

Mixture Study

A mixture can usually be defined by the presence of more than two alleles at a locus, the presence of a peak at a stutter position that is greater in percentage than typically observed in a single-source sample or even by the presence of significantly imbalanced alleles for a heterozygous genotype (Applied Biosystems, 2011).

Thus, this study intends to evaluate the behaviour of a certain multiplex kit with mixed samples. This is mostly important in more complex mixtures where a major and a minor contributor are present, where the distinction between the minor component and possible stochastic effects is very difficult. Therefore it is important to determine the limit of detection of the minor component at which a full profile may be guaranteed. It is common practice to use at least two known samples mixed at different concentrations in order to evaluate this parameter (Applied Biosystems, 2011, Qiagen, 2010).

Precision Study

Precision concept involves the determination of accurate and reliable genotypes over time. This study consists in measuring the base pair sizes for STR allele amplification products. All alleles should be situated within a +/- 0.5 bp window around the measured size for the corresponding allele in the allelic ladder. When sample alleles do not size within this reference window, the PCR product must be reanalysed in order to distinguish between a true off-ladder allele and a measurement error. Precision results are typically represented by the standard deviation values of the measured alleles in study and it allows for correct identification of a possible microvariant allele (non-

consensus allele). Thus, it should also be taken into account that sizing differences can occur, in the same instrument, due to a number of factors, such as type and concentration of the polymer, run temperature and other electrophoresis conditions (Applied Biosystems, 2011, Butler, 2005).

Degradation Study

This sort of study assumes that degraded DNA samples are a common finding in forensic genetic cases. The natural process of sample degradation usually comes with the decrease in size of DNA fragments that are available to be analysed, due to environmental exposure to various kinds of agents. Artificial degradation can always be simulated, using procedures that involve the action of DNases, the exposure to UV light or even by the process of sonification. Since degraded DNA samples are highly represented in routine forensic casework, in degradation studies, the ability of a certain set of loci to generate full and reliable profiles is evaluated (Coble and Butler, 2005).

Contamination Study

This is considered a crucial study, when a new STR profiling system is to be implemented. Its importance resides in the ability of generating an STR profile without contamination from an external source. In fact, being the next generation PCR kits highly sensitive, they could also be more susceptible to contamination, since very low levels of external DNA are now more expected to be detectable. This negative effect may arise from handling issues during sample collection, sample handling errors, pipetting errors and could result in incorrect assessments of samples and sample mixtures. To follow and evaluate this parameter, adequate controls, namely blank controls, should be used in all internal validation studies in order to be correctly monitored (Qiagen, 2010).

Reproducibility Study

In this sort of study the aim resides in evaluating and confirming that a same sample extracted from different sources or even samples from the same source (e.g. blood, semen, oral swab) should always have matching STR profiles (Butler, 2005).

2. BACKGROUND AND SPECIFIC AIMS

The routine casework and activity of IPATIMUP´s laboratory, in kinship and genetic identification domains, have nowadays an increased responsibility of an accredited space with strict measures of quality control, being evaluated by the annual participation in the Proficiency Testings (parentage and forensic), organized by GHEP-ISFG (Spanish and Portuguese Speaking Working Group of the International Society for Forensic Genetics) and in the triannual Proficiency Testing of CAP (College of American Pathologists), Parentage/Relationship Testing (PARF) Exercises of paternity and forensic challenges, organized by this society.

The current markers used on a routine basis in IPATIMUP´s laboratory are analysed through two multiplex systems, Identifiler Plus (Applied Biosystems) and Powerplex 16 HS (Promega), which amplify a total of 17 STRs and share 13 loci between them. In most parentage investigations, this set is informative enough for a sound conclusion of the kinships involved. However, in deficient paternities or in more complex relationships, this may not be true. If there is need for more genetic information, the laboratory can access to an “in-house” multiplex, composed of 4 loci (CD4, F13A01, FES and MBPB), which may aid in obtaining further informativeness. This kit was developed many years ago (Alves *et al*., 2004) with loci that are now rarely used by genetic forensic laboratories. As a consequence, participation in proficiency testing with these markers will not yield consensus, and so external quality control will not be accomplished. Therefore, the need for a new set of STRs became demanding, which also would preferably be more informative than the aforementioned “in-house” loci. Of course, there are other resources available which may be employed to increase informativeness depending on the case, namely autosomal Single Nucleotide Polymorphisms [SNPs; e.g. Indels, (Pereira *et al*., 2009)], Y-chromosome markers (e.g. Gusmão and Alves, 2005), X-chromosome STRs (e.g. Gusmão *et al*., 2012) and mitochondrial DNA sequences (e.g. Prieto *et al*., 2011). However, it is well known that autosomal STRs are the most informative markers in the majority of cases which allow for a higher power of discrimination.

On the other hand, since it is more and more common to have casework involving degraded DNA samples, it was necessary to implement new markers which would be amplified in smaller amplicons. This idea came from the fact that the new European Standard Set (ESS) of genetic markers were mostly consisting of mini-STRs and were

recently included in commercial kits together with other loci already analysed in IPATIMUP´s laboratory.

Therefore, considering the last recommendations from Europe, it was decided to implement these markers in routine casework, thus the necessity to carry out an internal validation study of a new generation kit of autosomal markers, the ESSplex Plus kit (Qiagen), containing the ESS loci. The choice for this particular commercial kit, from Qiagen, was due to our confidence in the consistent and robust results provided by Qiagen products, previously experienced in our laboratory, but especially due to its attractive price, in comparison to the other available choices.

Thus, the specific aims of this work were:

- Re-thinking the laboratory's strategy: the need for more informativeness (e.g. in complex kinships and deficient paternity cases) and evolution to the current European standards by incorporation and validation of a new additional set of European genetic markers in the Portuguese population. This includes a population genetic study for each new STR (allele frequency estimation, Hardy-Weinberg equilibrium test and parameters of forensic interest) as well as a segregation analysis.

- Internal validation of a next generation kit to be introduced in the laboratory's future routine. Since the actual scheme is based on the simultaneous use of Identifiler Plus (Applied Biosystems) and Powerplex 16 HS (Promega Corporation) as an internal quality control measure, the idea would be to replace one of these by the new kit, if its performance proved to be better.

- Increase the genetic information power relatively to degraded samples with the adoption of new mini-STRs (amplicons < 200bp), belonging to the new ESS.

- Contribute to the increase of quality control procedures at IPATIMUP's laboratory and taking the opportunity to validate a greater number of genetic markers at a lower cost and with higher power of discrimination, thus improving the power of the "in-house" database.

- Estimate specific thresholds, ratios and investigate about the next generation kits performances in order to make this study an auxiliary guide, to be consulted in future laboratory analysis.

3. MATERIAL & METHODS

3.1. Sampling strategy

The sampling process involved choosing an adequate set of samples to use in all processes of this work.

Since this work involved a population genetics analysis and a segregation study for the five new ESS loci, 370 DNA samples were selected, comprising 120 true trios (125 fathers, 125 mothers and 120 sons/daughters). These trios served as the basis for the segregation study, and all analyses performed for the population study included the typing of the fathers and mothers (250 unrelated individuals living in Portugal). All samples derived from these individuals were involved in prior paternity investigations and were thus already genetically investigated using the well-established PCR multiplex kits Powerplex® 16 HS kit (Promega) and AmpFlSTR Identifiler® kit (Applied Biosystems).

For the more specific internal validation studies, DNA samples were extracted for this particular purpose. They were collected from nine technical and administrative personnel from IPATIMUP Diagnostics, and different DNA sources were used for each individual: blood stains, total blood (Figure 9, a and b); different types of buccal swabs: Omni Swab by Whatman (Figure 10a), brush (Figure 10b) and cotton swab (Figure 10c).

Figure 9 - Two different types of blood sample: a) stain sample (FTA card) and b) total blood (liquid).

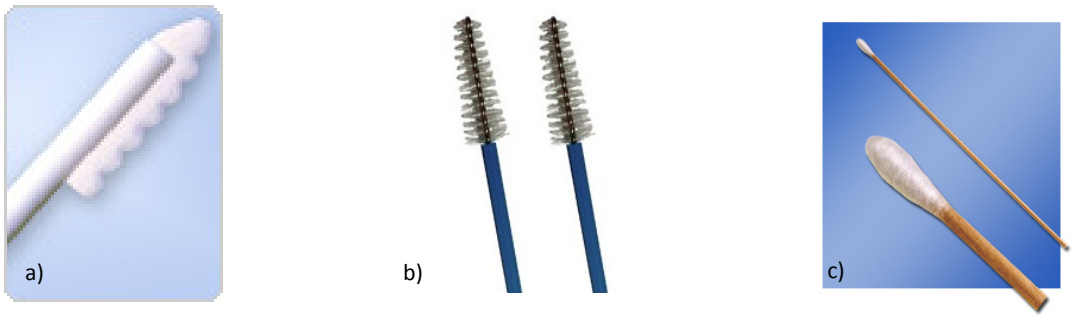


Figure 10 - Different models of swabs used in our study: a) Omni-Swab; b) Brush swab and c) Cotton swab.

3.2. Technical procedures

3.2.1. DNA extraction

All collected samples underwent a standard extraction protocol (the Chelex resin method) usually used in the laboratory routine and adapted from (Lareu *et al*., 1994). The preparation of samples for extraction involved two main procedures, depending on the type of biological material: blood or buccal swabs.

In the case of blood stains on FTA type paper, the procedure was initiated by cutting a square with approximately 3mm x 3mm to a 1.5ml tube. Otherwise, if it were whole blood, 10µl was added to a 1.5ml tube. In the next step, 1ml of H2O was added and the tubes were incubated for 30 minutes, shaking occasionally. After that, samples were centrifuged at 18620 xg (Microcentrifuge Hettich Mikro 200, 14000rpm) for 4 minutes and the supernatant discarded by pipetting most of the liquid. Afterwards, 200µl of a 5% Chelex solution (Chelex 100, 200-400 mesh) was added, with a 1000µl pipette, in constant agitation. After mixing the samples in a vortex shaker, they were incubated at 100ºC, for 8 minutes. Finally the tubes were stirred again in a vortex and centrifuged at 18620 xg (Microcentrifuge Hettich Mikro 200, 14000rpm) for 4 minutes. The samples were then temporarily stored in the fridge (4ºC) if they were to be immediately used. Otherwise they would be stored at -20ºC for subsequent utilizations.

Concerning swab samples, there are some differences in pre-extraction treatment, depending on the type of swab. The cotton swab and the Omni Swab have similar procedures. The extremities of both types of swabs were sectioned to the respective 1.5ml tubes and 200µl of 5% Chelex solution were added directly into the tubes in constant agitation, with a 1000µl pipette. After that, the samples suffered a small vortex shake and were incubated at 100ºC, for 8 minutes. Finally, they were centrifuged at 18620 xg (Microcentrifuge Hettich Mikro 200, 14000rpm) for 4 minutes and were then temporarily stored in the fridge (4ºC). Concerning brush swabs, the main difference in the extraction method resides in the fact that the brush swab with the buccal cells is taken directly to a 1.5ml tube with ethanol (96%). So first, the tubes had to be centrifuged at 14000 rpm for 10 minutes. The excess of ethanol was discarded and the tubes with the cellular material were placed, opened, in a hotplate at 70ºC, for 15 to 20 minutes, until all ethanol had evaporated. After this, the procedures are the same comparatively to the other types of swabs: 200µl of 5% Chelex solution added directly into the tubes, in constant agitation with a 1000µl pipette. All samples suffered a small

vortex shake and were incubated at 100ºC for 8 minutes. They were centrifuged at 18620 xg (Microcentrifuge Hettich Mikro 200, 14000rpm) for 4 minutes and were then temporarily stored in the fridge (4ºC) (Alves, 2011).

3.2.2. DNA quantification

DNA samples used for internal validation studies, were quantified by using a Real-Time PCR method (RT-PCR), namely the Investigator Quantiplex kit (Qiagen), generally according to the manufacturer's instructions (Qiagen, 2011a). The reaction was run on the 7500 Fast Real-Time PCR System (Applied Biosystems).

This kind of analysis requires the preparation of fresh serial dilutions of the Control DNA Z1 sample with the QuantiTect Nucleic Acid Dilution Buffer, both supplied by the manufacturer, according to Table 4 (Qiagen, 2011a).

Table 4 - Serial dilutions of the Control DNA Z1 for quantitation purposes (Qiagen, 2011a).

Serial dilution of Control DNA Z1	Control DNA Z1	QuantiTect Nucleic Acid Dilution Buffer
20 ng/µl	Undiluted DNA	–
5 ng/µl	10 µl	30 µl
1.25 ng/µl	10 µl	30 µl
0.3125 ng/µl	10 µl	30 µl
0.078125 ng/µl	10 µl	30 µl
0.01953125 ng/µl	10 µl	30 µl
0.0048828125 ng/µl	10 µl	30 µl

Thus, standards were serially diluted across a DNA concentration range of 20 ng/µl to 0.0048828125 ng/µl. The standard curve, consisting of 7 concentration points, is always critical for more accurate analysis.

PCR amplification conditions were first tested in two different final reaction volumes: the recommended 25 µl and the optimized 10 µl. In this way, 2.0 µl of DNA sample was combined with 23 µl of PCR Master Mix (Reaction Mix plus Primer Mix) for a total reaction volume of 25 µl (Qiagen, 2011a), and tested against 0.8 µl of DNA sample

with 9.2µl of Master Mix for a total reaction volume of 10 µl. The latter method was chosen and applied to the remaining samples.

The reaction was prepared in plates with 96 wells, and firmly covered with appropriate sealing film. An example of a plate setup of reactions on the Applied Biosystems 7500 Fast Real-Time PCR System is represented in Table 5 (Qiagen, 2011a). Samples to be quantified were loaded in triplicate and a blank (NTC) was always included.

Table 5 - Graphic representation of a possible plate setup for Real Time-PCR reaction using Quantiplex Kit (Qiagen, 2010, Qiagen, 2011a).

Well contents

	1	2	3	4	5	6	7	8	9	10	11	12
A	20	20	5	5	1.25	1.25	0.3125	0.3125	0.0781	0.0781	0.0195	0.0195
B	0.0049	0.0049	NTC	NTC	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
C	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
D	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
E	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
F	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
G	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK
H	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK	UNK

It is recommended that the standard curve be made from two replicates of each DNA Z1 concentration, and this was always carried out. Note that NTC (No Template Control) samples should be included in each quantification run in order to detect contamination. The NTC samples contain the reagent mix and the QuantiTect Nucleic Acid Dilution Buffer. As a control measure, it was also included a DNA sample with known quantity: XY13 (2 ng/µl) and 9947A (0.1 ng/µl) were the commercially available cell lines included in this study.

Before starting the RT-PCR, parameters and details must be included in the 7500 Fast Real-Time PCR System software (Applied Biosystems, 2010), according to the manufacturer's instructions. The PCR amplification conditions for quantitation were as follows: initial hold at 95 ºC for 1 min, followed by 40 cycles of denaturation at 95ºC for 5 sec and annealing at 60ºC for 25 sec.

After the RT-PCR occurred (± 40 min.), the results were analysed by the 7500 Fast Real-Time PCR System software (Applied Biosystems, 2010) using the recommendations included in the Quantiplex User´s Manual (Qiagen, 2011a).

3.2.3. DNA amplification

Taking into account that the main goal was to simultaneously increase the genetic informativeness and make the most economical choice, the option for ESSplex Plus Kit (Qiagen) made sense for application in the laboratory's routine. This Next Generation Kit is already an optimized version of the ESSplex Kit (Qiagen) whose chemical features were adjusted: the fast cycling technology enables amplification in just 90 minutes, with a higher sensitivity when compared with the ESSplex version.

Nevertheless, another next generation Kit was also tested: the NGM Kit (Applied Biosystems) was investigated for concordance purposes and also in other validation studies.

Apart from this, Identifiler Plus® kit (Applied Biosystems) and Powerplex® 16 HS kit (Promega) were also tested as a means of comparison between performances of routinely used kits and the next generation ones.

Since the four multiplex systems have different components and comprise specific methods, the following section describes the PCR procedures involved for each commercial kit.

Investigator® ESSplex Plus Kit

The characteristics of the genetic markers (loci), that compose this forensic kit, are the following (Table 6):

Table 6 - Specific information about each locus of Investigator ESSplex Plus Kit (Qiagen, 2010).

Locus	GenBank® accession number	Repeat motif of the reference allele	Chromosomal mapping
Amelogenin X	M55418	–	Xp22.1-22.3
Amelogenin Y	M55419	–	Yp11.2
D1S1656	NC_000001.9	$[TAGA]_{16}$ $[TGA][TAGA][TAGG]_1[TG]_5$	1q42
D2S441	AL079112	$[TCTA]_{12}$	2p14
D2S1338	G08202	$[TGCC]_6[TTCC]_{11}$	2q35
D3S1358	11449919	TCTA $[TCTG]_2$ $[TCTA]_{15}$	3p25.3
D8S1179	G08710	$[TCTA]_{12}$	8q23.1-23.2
D10S1248	AL391869	$[GGAA]_{13}$	10q26.3
D12S391	G08921	$[AGAT]_5$ GAT $[AGAT]_7$ $[AGAC]_6$ AGAT	12p13.2
D16S539	G07925	$[GATA]_{11}$	16q24.1
D18S51	L18333	$[AGAA]_{13}$	18q21.3
D19S433	G08036	AAGG [AAAG] AAGG TAGG $[AAGG]_{11}$	19q12
D21S11	AP000433	$[TCTA]_4$ $[TCTG]_6$ $[TCTA]_3$ TA $[TCTA]_3$ TCA $[TCTA]_2$ TCCATA $[TCTA]_{11}$	21q21.1
D22S1045	AL022314	$[ATT]_{14}$ ACT $[ATT]_2$	22q12.3
FGA (FIBRA)	M64982	$[TTTC]_3$ TTTTTTCT $[CTTT]_{13}$ CTCC $[TTCC]_2$	4q28.2
TH01 (TC11)	D00269	$[TCAT]_9$	11p15.5
vWA	M25858	TCTA $[TCTG]_4$ $[TCTA]_{13}$	12p13.31

The recommended volumes of the different PCR components per reaction are indicated in Table 7 (Qiagen, 2010). The recommended amount of input DNA for ESSplex Plus is 0.5 ng (Qiagen, 2011b).

Table 7 - Components and respective volumes of the PCR reaction (Qiagen, 2010).

Component	Volume per reaction
Fast Reaction Mix	7.5 µl
Primer Mix	2.5 µl
Nuclease-free water (added in step 4)	Variable
Template DNA (added in step 4)	Variable
Total volume	**25 µl**

The amplification protocol for the ESSplex Plus Kit followed the manufacturer's instructions with the exception of a reduced PCR volume of 10 µl, in a GeneAmp® PCR system 9700 (Applied Biosystems). This is routine policy in the laboratory for lowering the costs in reagents and other materials. To be sure that this reduction does not hamper STR amplification and evaluation, both volumes (10 µl and 25 µl) were independently tested. There were no evident differences between 10 µl and 25 µl reactions (Qiagen, 2011b).

The same PCR conditions were always used for this next generation kit (Qiagen, 2010) and consisted of the following (Table 8):

Table 8 - Standard cycling program recommended for ESSplex Plus Kit (Qiagen, 2010).

Temperature	Time	Number of cycles
95°C (hot-start to activate DNA polymerase)	5 min	–
96°C	10 s	
61°C	120 s	30 cycles
10°C	∞	–

After this process, the amplified samples were stored at -20º C, protected from light, or proceeded directly to electrophoresis. For each PCR reaction that was undertaken, a positive control (cell line XY13, supplied by the manufacturer) and a negative control (PCR reagents plus sterile water) were analysed.

AmpFISTR® NGM™ Kit

Concerning the genetic composition of NGM Kit, it amplifies exactly the same genetic markers present in ESSplex Plus Kit.

Relatively to the amplification reaction, the volume of each component is indicated in Table 9. The recommended input DNA for NGM Kit is 1 ng (Applied Biosystems, 2011).

Table 9 - Components and respective volumes of the PCR reaction (final volume of 25µl) (Applied Biosystems, 2011).

DNA sample	Volume per reaction (µL)
AmpFℓSTR® NGM™ Master Mix	10.0
AmpFℓSTR® NGM™ Primer Set	5.0

Once again, the amplification protocol for the NGM Kit followed the manufacturer's instructions, but reducing the PCR final volume to 10 µl, in a GeneAmp® PCR system 9700 (Applied Biosystems). Both volumes (10 µl and 25 µl) were independently tested and there were no evident differences between them.

A negative and a positive control, namely the cell line 007 supplied by the manufacturer, were always added in each PCR reaction.

Relatively to the thermal cycling conditions (Table 10), the following program was used:

Table 10 - Standard conditions of thermal cycling program, for NGM Kit (Applied Biosystems, 2011).

Initial incubation step	Cycle (29 cycles)		Final extension	Final hold
	Denature	Anneal		
HOLD	CYCLE		HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 3 min	60 °C 10 min	4 °C ∞

After this process, the amplified samples were stored at -20º C, protected from light, or proceeded directly to electrophoresis.

Identifiler® Plus Kit

Since this internal validation study aims to evaluate the performance of a new generation kit comparatively to the usually used in the laboratory's routine, the Identifiler® Plus Kit was also analysed for comparison purposes. In terms of genetic markers, its composition is different from the previous Next Generation Kits.

The characteristics of the genetic markers (loci) that compose this forensic kit are shown in Table 11 (Applied Biosystems, 2012).

Table 11 - AmpFlSTR® Identifiler® Plus Kit loci, alleles, chromosome location and 9947A positive control (Applied Biosystems, 2012).

Locus designation	Chromosome location	Alleles included in Identifiler® Plus Allelic Ladder	Dye label	Control DNA 9947A
D8S1179	8	8, 9 10, 11, 12, 13, 14, 15, 16, 17, 18, 19	6-FAM™	13‡
D21S11	21q11.2-q21	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38		30§
D7S820	7q11.21-22	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 11
CSF1PO	5q33.3-34	6, 7, 8, 9, 10, 11, 12, 13, 14, 15		10, 12
D3S1358	3p	12, 13, 14, 15, 16, 17, 18, 19	VIC®	14, 15
TH01	11p15.5	4, 5, 6, 7, 8, 9, 9.3, 10, 11, 13.3		8, 9.3
D13S317	13q22-31	8, 9, 10, 11, 12, 13, 14, 15		11#
D16S539	16q24-qter	5, 8, 9, 10, 11, 12,13, 14, 15		11, 12
D2S1338	2q35-37.1	15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28		19, 23
D19S433	19q12-13.1	9, 10, 11, 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2	NED™	14, 15
vWA	12p12-pter	11,12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24		17, 18
TPOX	2p23-2per	6, 7, 8, 9, 10, 11, 12, 13		8‡‡
D18S51	18q21.3	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27		15, 19
Amelogenin	X: p22.1-22.3 Y: p11.2	X, Y	PET®	X
D5S818	5q21-31	7, 8, 9, 10, 11, 12, 13, 14, 15, 16		11§§
FGA	4q28	17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 26.2, 27, 28, 29, 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2		23, 24

Concerning the amplification reaction, the following table (Table 12) shows the volumes of the respective reagents, for a final volume of 25µl. The recommended input DNA for Identifiler Plus Kit is 1 ng (Applied Biosystems, 2012).

Table 12 - Components and respective volumes of the PCR reaction (final volume of 25µl) (Applied Biosystems, 2012).

DNA sample	Volume per reaction (µL)
AmpFℓSTR® Identifiler® Plus Master Mix	10.0
AmpFℓSTR® Identifiler® Plus Primer Set	5.0

In our studies, the final PCR volume was adapted to 10 µl since both volumes (10 µl and 25 µl) were independently tested and there were no evident differences between them. A negative and a positive control (cell line 9947A supplied by the manufacturer) were added in each PCR reaction.

Concerning thermal cycling conditions (Table 13), the program used was the following:

Table 13 - Standard conditions of thermal cycling program for Identifiler® Plus Kit; 28 cycles was used for all the tests performed (Applied Biosystems, 2012).

Initial incubation step	Cycle (28 or 29 cycles‡)		Final extension	Final hold
	Denature	Anneal/Extend		
HOLD	CYCLE		HOLD	HOLD
95 °C 11 min	94 °C 20 sec	59 °C 3 min	60 °C 10 min	4 °C ∞

After this process, the amplified samples were stored at -20º C, protected from light, or proceeded directly to electrophoresis.

PowerPlex® 16 HS Kit

Since PowerPlex® 16 HS Kit is the other multiplex kit used routinely in casework, it was also analysed for comparison purposes. In Table 14, the PowerPlex® 16 HS System Locus-Specific Information is represented (Promega Corporation, 2009 - 2012).

Table 14 - The PowerPlex® 16 HS System Locus-Specific Information (Promega Corporation, 2009 - 2012).

STR Locus	Label	Chromosomal Location	GenBank® Locus and Locus Definition	Repeat Sequence[1] 5´→3´
Penta E	FL	15q	NA	AAAGA
D18S51	FL	18q21.3	HUMUT574	AGAA (22)
D21S11	FL	21q11–21q21	HUMD21LOC	TCTA Complex (22)
TH01	FL	11p15.5	HUMTH01, human tyrosine hydroxylase gene	AATG (22)
D3S1358	FL	3p	NA	TCTA Complex
FGA	TMR	4q28	HUMFIBRA, human fibrinogen alpha chain gene	TTTC Complex (22)
TPOX	TMR	2p24–2pter	HUMTPOX, human thyroid peroxidase gene	AATG
D8S1179	TMR	8q24.13	NA	TCTA Complex (22)
vWA	TMR	12p13.31	HUMVWFA31, human von Willebrand factor gene	TCTA Complex (22)
Amelogenin[2]	TMR	Xp22.1–22.3 and Y	HUMAMEL, human Y chromosomal gene for Amelogenin-like protein	NA
Penta D	JOE	21q	NA	AAAGA
CSF1PO	JOE	5q33.3–34	HUMCSF1PO, human c-fms proto-oncogene for CSF-1 receptor gene	AGAT
D16S539	JOE	16q24.1	NA	GATA
D7S820	JOE	7q11.21–22	NA	GATA
D13S317	JOE	13q22–q31	NA	TATC
D5S818	JOE	5q23.3–32	NA	AGAT

Concerning the PCR Amplification Mix for the PowerPlex® 16 HS System, the information is contained in Table 15:

Table 15 - PCR Amplification Mix for the PowerPlex® 16 HS System (Promega Corporation, 2009 - 2012).

Water, Amplification Grade	to a final volume of 25.0µl
PowerPlex® HS 5X Master Mix	5.0µl
PowerPlex® 16 HS 10X Primer Pair Mix	2.5µl
template DNA (0.5–1ng)[2,3]	up to 17.5µl
total reaction volume	**25µl**

The same adoption was made for this multiplex kit concerning the PCR final volume: 10 µl instead of the recommended 25 µl. A negative and a positive control (cell line 9947A supplied by the manufacturer) were added in each PCR reaction.

Relatively to the 9700 thermal cycler protocol for the PowerPlex® 16 HS System Kit, the parameters are represented in Figure 11 (Promega Corporation, 2009 - 2012).

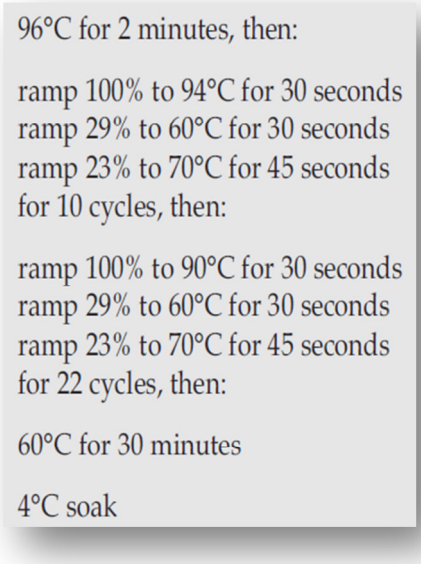


Figure 11 - Standard conditions of thermal cycling program for PowerPlex® 16 HS System Kit (Promega Corporation, 2009 - 2012).

After the thermal cycling reaction was complete, the amplified samples were stored at -20ºC, protected from light, or proceeded directly to electrophoresis.

3.2.4. Fragment detection and analysis

All the amplification products were separated and detected on an ABI Prism 3130 Genetic Analyser (Applied Biosystems). Genotyping was undertaken using the GeneMapper® ID Software v.3.2 (Applied Biosystems, 2005) by comparison to the corresponding allelic ladder supplied in each kit. All the multiplex kits panels, bins and analysis methods were obtained from the respective companies.

Table 16 - Structure of the four multiplexes tested regarding size standard, electrophoresis conditions, amount of input DNA and allelic ladder. The * symbol means an adaptation in injection time from 5 seconds to 10.

	Investigator® ESSplex Plus Kit	AmpFISTR® NGM™ Kit	Identifiler® Plus Kit	PowerPlex® 16 HS Kit
Size Standard	550 BTO	GeneScan 500 LIZ	GeneScan 500 LIZ	ILS 600
Electrophoresis Conditions	Table 17	*	*	*
Input DNA	0.5 µl	0.5 µl	0.5 µl	0.5 µl
Allelic Ladder	1.2 µl	1.2 µl	1.2 µl	1.2 µl

Regarding electrophoresis conditions (Table 16), the information concerning Investigator® ESSplex Plus Kit is described in Table 17 (Qiagen, 2010). The other multiplexes (AmpFISTR® NGM™ Kit, Identifiler® Plus Kit and PowerPlex® 16 HS Kit) have exactly the same electrokinetic parameters that were enunciated for ESSplex Plus Kit, but an adapted injection time from 5 seconds to 10 was undertaken (Applied Biosystems, 2011, Applied Biosystems, 2012, Promega Corporation, 2009 - 2012).

Regarding sample preparation, all mixes were made at the same proportions: a 10 µl mixture at 55:1 proportion: 825 µl Formamide *Hi-Di* with 15 µl of size standard.

Table 17 - Run Module 3kV_10s_500bp for ABI 3130/3130xl and our respective internal parameters (Qiagen, 2010).

Parameter	Settings	Study values
Oven Temperature (°C)	Default	60
Poly Fill Volume	Default	4840
Current Stability (µA)	Default	5
Pre-Run Voltage (kV)	Default	15
Pre-Run Time (s)	Default	180
Injection Voltage (kV)	3.0	3
Injection Time (s)	10*	10
Voltage Number of Steps	Default	20
Voltage Step Interval	Default	15
Data Delay Time (s)	Default	60
Run Voltage (kV)	Default	15
Run Time (s)	1560†	1200

3.3. Validation studies

3.3.1. Population Study

For population genetic studies, between 243 and 246 unrelated individuals living in Portugal were genotyped using the Investigator® ESSplex Plus Kit (Qiagen, 2011b). These samples compose the current IPATIMUP´s database for genetic identification and kinship analysis and were previously analysed with Identifiler and Powerplex 16 systems.

Allele frequencies were estimated for each of the five new markers (D2S441, D22S1045, D12S391, D10S1248 and D1S1656) using GenePop (v.4.1.0) software (Rousset, 2010). This software also allowed for assaying the Hardy-Weinberg equilibrium through an exact test (Guo and Thompson, 1992). Forensic parameters such as expected and observed heterozygosity were calculated according to Nei (Nei, 1987), and polymorphic information content (PIC), power of discrimination (PD) an d power of exclusion (PE) were estimated based on formulas accessed in (Botstein *et al.*, 1980) for PIC and in (Fisher, 1951) for PD and PE. A comparison was also made between our sample (Portuguese population) and other European samples available in the literature. For this purpose, the Arlequin Software (Excoffier and Lischer, 2010) was used and evaluation of any significant deviations is discussed.

Moreover, a set of 120 true trios (mother, father and son/daughter) were typed in order to test Mendelian inheritance of these five new markers and detect phenomena such as mutations and silent alleles.

3.3.2. Concordance Study

For concordance purposes, 120 unrelated individuals living in Portugal, corresponding to the sampled sons and daughters, previously genotyped with Identifiler and Powerplex 16 kits, were genotyped with the two different Next Generation Kits: ESSplex Plus (Qiagen, 2011b) and NGM (Applied Biosystems, 2011) according to the protocols already described.

Table 18 - Loci sharing between the multiplex kits analysed.

	ESS	NGM	Powerplex 16 HS	Identifiler Plus
AMEL	✖	✖	✖	✖
TH01	✖	✖	✖	✖
D3S1358	✖	✖	✖	✖
VWA	✖	✖	✖	✖
D21S11	✖	✖	✖	✖
D16S539	✖	✖	✖	✖
D1S1656	✖	✖		
D19S433	✖	✖		✖
D8S1179	✖	✖	✖	✖
D2S1338	✖	✖		✖
D10S1248	✖	✖		
D22S1045	✖	✖		
D12S391	✖	✖		
FGA	✖	✖	✖	✖
D2S441	✖	✖		
D18S51	✖	✖	✖	✖
Penta E			✖	
TPOX			✖	✖
Penta D			✖	
CSF1PO			✖	✖
D7S820			✖	✖
D13S317			✖	✖
D5S818			✖	✖

This kind of study allows to evaluate if there is some discordant result for the same samples using different kits (comparison of markers shared between them, see Table 18), with different performances and primer design, and, if there exists, the detection of primer binding site mutations that cause silent alleles, or allele drop-out.

The following figure (Figure 12) represents the procedure designed for our study:

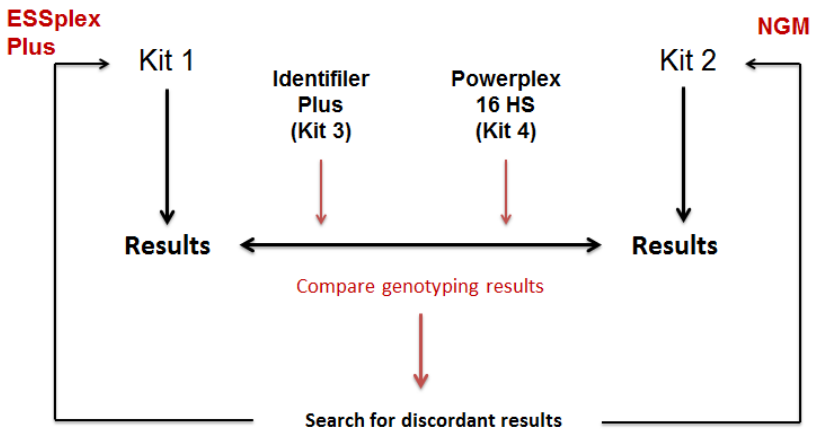


Figure 12 - Schematic representation of the concordance procedure.

3.3.3. Sensitivity Study, Stochastic Effects and Artifacts

3.3.3.1. Sensitivity

In order to evaluate the sensitivity of ESSplex Plus Kit and find the optimal concentration of input template DNA, serial dilutions of different types and sources of DNA were made: 6 blood samples stained on FTA type paper and total blood in EDTA tubes, and 9 swab type samples: Omni Swab (Whatman), brush and cotton, as previously described.

After extracting and quantifying the DNA obtained from each source, serial dilutions were performed, with a total of seven different concentrations, ranging from 0.5 ng/µl to 5 pg/µl.

The following table (Table 19) shows the concentrations of DNA used in the serial dilutions:

Table 19 - Example of the procedure designed to obtain a serial dilution of a DNA sample. The letter A represents the original samples and are indicated their respective values of initial concentration, which were used in this study.

Serial Dilution	Concentration(ng/µl)	Diluted DNA Amount(µl)	Water Amount(µl)
A Original DNA extract	[31-0.5]	-	[915-22.5]
B	0.5	15 of A	22.5
C	0.2	15 of B	15
D	0.1	15 of C	15
E	0.05	15 of D	15
F	0.02	15 of E	22.5
G	0.01	15 of F	15
H	0.005	15 of G	15

All the serial dilutions were quantified again to ensure greater reliability in our procedures.

Moreover, 5 representative samples were selected and were amplified with the Investigator ESSplex Plus, NGM, Identifiler Plus and Powerplex 16 HS kits, in order to include the range of DNA values that allows the evaluation of all possible DNA analysis effects: drop-out, drop-in, pull-up and full- profile. This analysis resulted from the same serial dilutions procedure that was established for the ESSplex Plus. Thus, PCR products were run on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems) and

all parameters used for electrophoresis were those previously described. Positive and negative controls were always included.

The different kind of samples and DNA concentrations used in this study also allowed to evaluate stochastic events and artefacts for the ESSplex Plus kit, namely stutter, pull-up, allele drop-in and drop-out and allele imbalance.

3.3.3.2. PHR evaluation

Regarding allele imbalance, the peak height ratio (PHR) was calculated (Figure 13) associated to each loci of the ESSplex Plus, in 33 samples.

$$\textit{Peak height ratio} = \frac{height\ of\ lower\ allele\ (RFU)}{height\ of\ higher\ allele\ (RFU)}$$

Figure 13 - Formula used to obtain the PHR values for each loci of the ESSplex Plus Kit.

3.3.3.3. Stutter evaluation

To calculate the stutter ratio for each locus of the ESSplex Plus Kit, 34 DNA samples from different sources (blood and swabs) were chosen in a range of [2 ng to 0.5 ng] as a means of contemplating the recommended DNA range (Qiagen, 2011b).

The proportion of the stutter product (Figure 14), relative to the main allele (Stutter Ratio), was calculated using the following index:

$$\textit{Stutter Ratio} = \frac{height\ of\ the\ stutter\ peak}{height\ of\ the\ main\ allele\ peak}$$

Figure 14 - Formula used for the calculation of the Stutter Ratio.

The 34 samples were selected by taking into account the allelic ranges for each locus of the ESSplex Plus kit, in the Portuguese population (Table 20).

Table 20 - Total allelic range for each genetic marker of ESSplex Plus Kit. In red are represented the alleles that were present in our selected samples, in the Portuguese population.

Loci - ESSplex Plus	Total Allelic Range
AMEL.	X/Y
TH01	4,5,6,7,8,9,9.3,10,10.3,13,13.3
D3S1358	9,10,11,12,13,14,15,16,17,18,19,20,21
Vwa	11,12,13,14,15,16,17,18,19,20,21,22
D21S11	24,24.2,25,26,26.2,27,28,28.2,29,29.2,30,30.2,31,31.2,32,32.2,33,33.2,34,34.2,35,36,36.2,37
D16S539	8,9,10,11,12,13,14,15
D1S1656	10,11,12,13,14,15,16,17,17.3,18.3,19.3
D19S433	6.2,10,11,12,12.2,13,13.2,14,14.2,15,15.2,16,16.2,17,17.2
D8S1179	7,8,9,10,11,12,13,14,15,16,17,18,19
D2S1338	16,17,18,19,20,21,22,23,24,25,26,27,28
D10S1248	10,11,12,13,14,15,16,17,18,19
D22S1045	10,11,12,13,14,15,16,17,18,19
D12S391	15,16,17,18,19,20,21,22,23,24,25,26
FGA	14,16,17,18,19,20,21,21.2,22,23,23.2,24,25,26,27,28,29,30,31.2,33,34,37.2,42.2,44.2,45.2,47.2,50.2
D2S441	8,9,10,11,11.3,12,13,14,15,16
D18S51	8,9,10,10.2,11,12,13,14,15,16,17,17.2,18,18.2,19,20,21,21.2,22,23,24,25,26,27,28

3.3.4. Mixture study

This study was conducted using a combination of samples from two individuals, both laboratory workers (1 male and 1 female). Blood samples were taken from these donors and two separate experiments were made in this mixture study. In the first test, female and male blood were mixed at increased quantities of female blood to constant quantity of male blood, at the ratios indicated in Table 21. In the second mixture experiment, the opposite was carried out, as indicated in Table 22. The mixtures were prepared across the ranges: 0:1, 1:1, 2:1, 5:1, 10:1, 15:1, 20:1. The 1:0 and 0:1 ratios provide the full and clean profile of each input sample used in the mixture analysis and act as the reference points.

Table 21 - Mixture test 1 design: constant male sample mixed with increased female sample quantities.

Mixture test 1		
Ratio	µl of Female blood	µl of Male blood
1:0	-	15
1:1	15	15
1:2	30	15
1:5	75	15
1:10	150	15
1:15	225	15
1:20	300	15

Table 22 - Mixture test 2 design: constant female sample mixed with increased male sample quantities.

Mixture test 2		
Ratio	µl of Female blood	µl of Male blood
1:0	15	-
1:1	15	15
1:2	15	30
1:5	15	75
1:10	15	150
1:15	15	225
1:20	15	300

The reference profiles from the two individuals tested are displayed in Table 23.

Table 23 - Reference profiles for Mixture study: Z codes are related with the internal code that is used in our laboratory.

Locus	Z 7607	Z 7451
	Male	Female
AMEL	X/Y	X
TH01	6/8	6/7
D3S1358	17/18	15
vWA	16/19	15/19
D21S11	28/32	29/30
D16S539	11/13	11/12
D1S1656	11/15.3	16/17.3
D19S433	14	14/15
D8S1179	13/14	13/15
D2S1338	16/19	19/23
D10S1248	13/16	15/16
D22S1045	14/15	15
D12S391	17/18	22/23
FGA	23/26	19/21
D2S441	10/11	11/11.3
D18S51	13/16	17/18

Therefore, for each mixture ratio prepared in 1.5ml tubes, 30 µl of the mixed blood was spotted on an FTA type paper. The DNA was then extracted and quantified by RT-PCR, using the Investigator Quantiplex Kit (Qiagen), and then were diluted to 0.5 ng/µl, which corresponds to the input DNA value recommended by Qiagen.

The PCR amplification design for the mixture study is identical to that of the sensitivity study, and was made according to the technical features of the Investigator® ESSplex Plus Kit, with the previously mentioned adjustments. After the PCR amplification step, samples from both mixture tests were loaded onto the ABI PRISM 3130 Genetic Analyser (Applied Biosystems) for fragment detection and data analysis using GeneMapper® ID (v.3.2) Software.

It was also estimated the proportion of contributors present in a certain mixture (Figure 15). The formula used to obtain this mixture ratio, per loci, was:

$$Mixture\ Ratio = \frac{sum\ of\ small\ peaks\ height}{sum\ of\ higher\ peaks\ height}$$

Figure 15 - Formula used in the calculation of Mixture Ratio.

3.3.5. Precision study

In this study, 33 allelic ladder injections were pooled together to evaluate the precision of the Investigator® ESSplex Plus Kit on the ABI PRISM 3130 Genetic Analyser. The precision was measured by calculating the standard deviation of the base pair size per allele, using the values obtained for each ladder, exported from GeneMapper® ID Software (Applied Biosystems, 2005) to the statistical software Microsoft Excel 2010. The mean sizes for all the alleles in each run were calculated and the loci with the lowest and highest values of deviation were also identified (Qiagen, 2010, Qiagen, 2012).

Also, evaluation of precision was done under controlled conditions with 22 ladder injections (time, room temperature and electrophoresis parameters, among others), and under varying conditions through time, by analysing a total of 11 ladder injections.

3.3.6. Degradation study

Since the UV-light exposure is a simple and fast method for artificially degrading DNA, this was the method of choice for our internal validation study (Bender *et al.*, 2004, Thacker *et al.*, 2006).

We based our study in previously described procedures (Tamariz *et al.*, 2006, Thacker *et al.*, 2006), taking into account the type of UV-light (UV-C) and the respective wavelength spectrum (100 – 280 nm). For this study, a Mini-V/PCR vertical laminar flow bench (Telstar), containing a 254 nm wavelength UV lamp with 15 watts, was used.

The procedure undertaken was as follows:

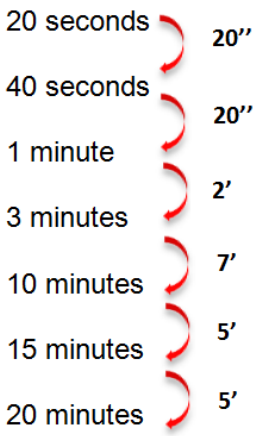
I. A DNA sample, previously quantified and diluted to the recommend value (0.5ng/µl), was chosen;

II. 7 tissue slides were cleaned and dried with ethanol;

III. 10 µl of DNA sample were placed in each slide, approximately in the centre;

IV. All 7 slides were placed on a support at an approximate distance of 10cm from the UV-light lamp, inside the cabinet. All slides were previously identified with the sample code and time of exposure;

V. After turning the UV light on, each slide suffered different times of UV exposure. Each slide was removed after turning the UV-light off when the respective period of exposure was finished. UV exposure time intervals were the following:



VI. After all the samples were removed, the slides were left to dry completely. 10 µl of sterilized water were added to each dried stain and mixed thoroughly to rehydrate the samples;

VII. The 10 µl from each slide were transferred to previously identified tubes, for future use;

PCR reactions and fragment analysis were undertaken as previously described.

3.3.7. Contamination study

The potential for contamination of a particular multiplex in laboratory routine work was evaluated by a contamination study, which comprises the range of tests included in an internal validation.

In this study all the negative (or blank) controls (composed by the mixture of reagents and sterilized water) used in all internal validation studies (population analysis, concordance, sensitivity, mixture, and degradation) were analysed. The methodology used consisted in the detection of any amplified PCR product in these blank controls, using the Investigator® ESSplex Plus Kit (N=9) (Qiagen), the NGM (N=8) (Applied Biosystems), the Identifiler Plus (N=19) (Applied Biosystems) and the Powerplex HS 16 (N=11) (Promega Corporation).

4. RESULTS AND DISCUSSION

4.1. Population Study

A total of 250 unrelated individuals (125 females and 125 males) living in Portugal were sampled to be genotyped for the 15 STR loci (D10S1248, D22S1045, D2S441, D1S1656, D12S391, D2S1338, D3S1358, D8S1179, D16S539, D18S51, D19S433, D21S11, FGA, TH01, vWA) included in the ESSplex Plus amplification kit.

The population study was focused on the five new loci (D2S441, D22S1045, D10S1248, D12S391 and D1S1656), since data was already available for the remaining 10 loci (Amorim *et al.*, 2006).

Allelic frequencies for the five new genetic autosomal markers are shown in Table 24. The p-values results confirmed that the frequencies are in Hardy - Weinberg equilibrium ($p > 0.05$), in our Portuguese sample (Table 24).

Some forensic parameters were also estimated: the expected heterozygosity values are comprised in the range of 0.7021 – 0.8979, being the highest value associated to the D1S1656 marker (Table 24); relatively to the power of discrimination values, they are situated in the range of 0.8603 – 0.9800, being all values superior to 85%. The highest value for this forensic parameter is also given by the D1S1656 marker and the lowest one by D22S1045 (Table 24).

Moreover, it is also possible to observe that the power of exclusion range is between 0.4574 – 0.7897, with the highest value also belonging to D1S1656 and the lowest one to D22S1045 (Table 24).

Overall it is observed that D1S1656 is the most informative marker, since it also shows the highest polymorphic information content with an approximate value of 89%. This forensic parameter is always above 65% for this set of new forensic genetic markers (Table 24).

Table 24 - Allele frequencies and forensic parameters of interest for the five STRs D1S1656, D2S441, D10S1248, D12S391 and D22S1045, in our Portuguese sample.

Allele	D1S1656	D2S441	D10S1248	D12S391	D22S1045
9	-	0.0020	0.0020	-	-
10	-	0.2082	-	-	0.0041
11	0.0535	0.3122	0.0020	-	0.1163
11.3	-	0.0714	-	-	-
12	0.1296	0.0469	0.0369	-	0.0061
13	0.0700	0.0245	0.2725	-	0.0041
14	0.0967	0.2878	0.3607	-	0.0286
14.3	0.0021	-	-	-	-
15	0.1502	0.0408	0.1742	0.0203	0.3347
15.3	0.0741	-	-	-	-
16	0.1091	0.0061	0.1127	0.0224	0.4041
16.3	0.0556	-	-	-	-
17	0.0432	-	0.0328	0.0833	0.098
17.3	0.1523	-	-	0.0305	-
18	0.0103	-	0.0061	0.1951	-
18.3	0.0412	-	-	0.0285	-
19	-	-	-	0.1037	0.0041
19.1	-	-	-	0.0020	-
19.3	0.0123	-	-	0.0244	-
20	-	-	-	0.1260	-
21	-	-	-	0.0976	-
22	-	-	-	0.1199	-
23	-	-	-	0.0874	-
24	-	-	-	0.0407	-
25	-	-	-	0.0122	-
26	-	-	-	0.0061	-
N	243	245	244	246	245
Obs.He.	0.8971	0.7429	0.7500	0.8618	0.7265
Exp.He.	0.8979	0.7683	0.7517	0.8935	0.7021
P-value	0.7417	0.4182	0.8072	0.3323	0.3774
SE	0.0067	0.0072	0.0088	0.0092	0.0088
PIC	0.8869	0.7306	0.7112	0.8822	0.6505
PD	0.9800	0.9095	0.8986	0.9788	0.8603
PE	0.7897	0.5536	0.5283	0.7828	0.4574

N=number of individuals; Obs.He. =observed heterozygosity; Exp.He. =expected heterozygosity; P-value=probability values of the Hardy-Weinberg equilibrium exact test (50 batches and 10000 interactions); SE= standard error of P-value; PIC=polymorphic information content; PD= power of discrimination; PE=power of exclusion.

Transmission studies of the five new STRs carried out in a set of 120 true trios (mother, father and son/daughter) confirmed Mendelian inheritance. Through this analysis, there was no observation of mutations or silent alleles. Even between profiles generated by the four different multiplex kits used was there any observation of such phenomena.

A genetic comparison between our Portuguese sample and other European samples already available in the literature was also carried out, in order to assess any significant deviations that may exist, concerning the five new markers.

D1S1656

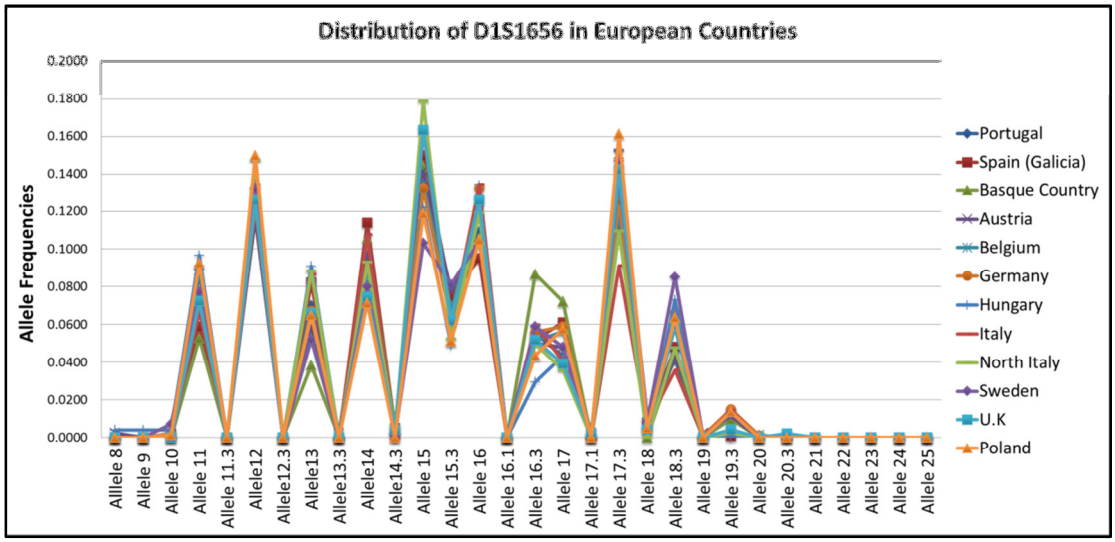


Figure 16 - Graphic representation of the allele frequency distribution for the D1S1656 marker, in European samples.

Regarding the D1S1656 marker, it is possible to observe, in Figure 16, a homogeneous distribution of the allele frequencies across European countries [Spain(Galicia) (Formoso *et al.*, 2012), Basque Country (Yurrebaso *et al.*, 2011), Austria (Dognaux *et al.*, 2011), Belgium (Berti *et al.*, 2010), Germany (Seider *et al.*, 2010), Hungary (Martín *et al.*, 2007), Italy (Berti *et al.*, 2010), North Italy (Cortellini *et al.*, 2011), Sweden (Albinsson *et al.*, 2011), U.K. (Tucker *et al.*, 2011a), Poland (Hatzer-Grubwieser *et al.*, 2011)]. So, in this case, the comparison of our Portuguese sample with other European samples revealed no significant allele frequency differences.

However, some statistical differences were observed, concerning this forensic genetic marker, between Sweden (Albinsson *et al.*, 2011) and Italy (Previderè *et al.*, 2011), and between Sweden (Albinsson *et al.*, 2011) and North Italy (Cortellini *et al.*, 2011) samples (Figure 17).

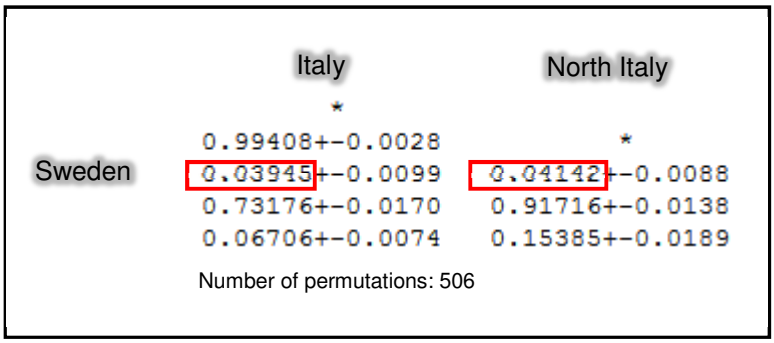


Figure 17 - Statistical deviations detected for the D1S1656 genetic marker between European samples.

However, after applying the Bonferroni correction for multiple tests ($P < 0.05 / 5 = 0.01$), the data suggests that no significant deviations should be considered.

D2S441

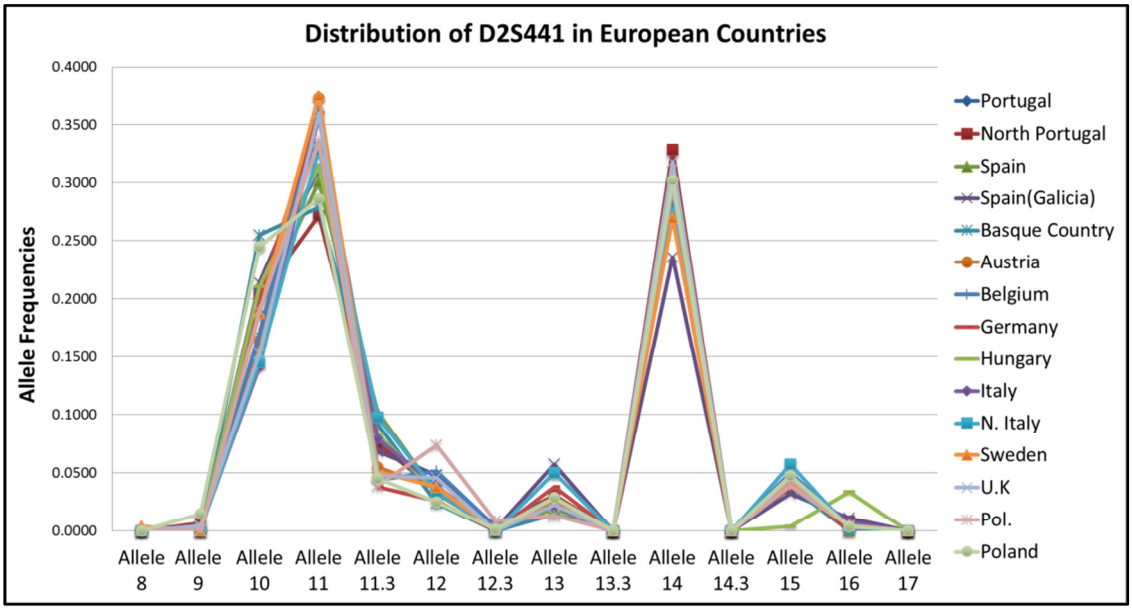


Figure 18 - Graphic representation of the allele frequency distribution for the D2S441 marker, in European samples.

In respect to the D2S441 marker, the allele frequency distribution is represented in Figure 18, for the available European data [North Portugal (Lagoa *et al.*, 2008), Spain (Martín *et al.*, 2007), Spain(Galicia) (Formoso *et al.*, 2012), Basque Country (Yurrebaso *et al.*, 2011), Austria (Dognaux *et al.*, 2011), Belgium (Berti *et al.*, 2010), Germany (Seider *et al.*, 2010), Hungary (Martín *et al.*, 2007), Italy (Berti *et al.*, 2010), North Italy

(Cortellini *et al*., 2011), Sweden (Albinsson *et al*., 2011), U.K. (Tucker *et al*., 2011a), Pol. (Reichert and Pawlowski, 2009), Poland (Hatzer-Grubwieser *et al*., 2011)]. Also, in this case the comparison of our Portuguese sample with other European samples revealed, apparently, no significant allele frequency differences.

Nevertheless, some statistical differences were observed between Poland (Reichert and Pawlowski, 2009) and Sweden (Albinsson *et al*., 2011), Austria (Hatzer-Grubwieser *et al*., 2011), Belgium (Dognaux *et al*., 2011), Italy (Berti *et al*., 2010), North Italy (Cortellini *et al*., 2011) and U.K. (Tucker *et al*., 2011a) and also between North Portugal (Lagoa *et al*., 2008) and Sweden (Albinsson *et al*., 2011) (Figure 19).

However, after applying Bonferroni correction ($P < 0.05 / 5 = 0.01$), no deviations were considered statistically significant.

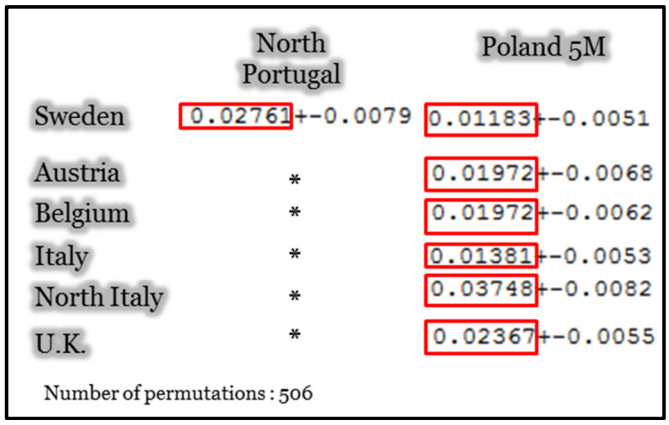


Figure 19 - Statistical deviations detected for the D2S441 genetic marker between European samples.

D10S1248

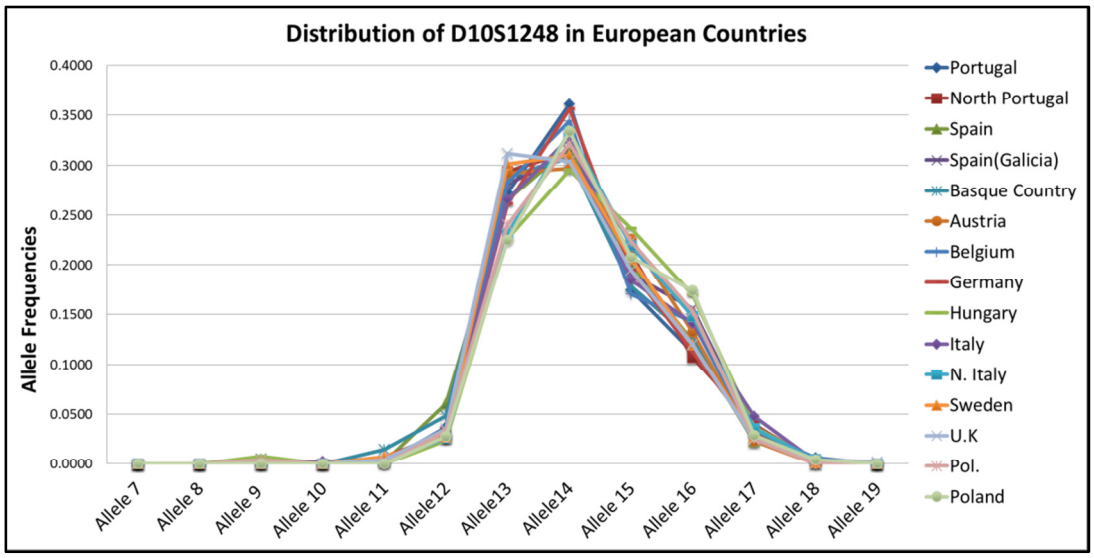


Figure 20 - Graphic representation of the allele frequency distribution for the D10S1248 marker, in European samples.

Relatively to the D10S1248 marker, in Figure 20, it is possible to observe the distribution of the allele frequencies between European countries [North Portugal (Lagoa *et al.*, 2008), Spain (Martín *et al.*, 2007), Spain(Galicia) (Formoso *et al.*, 2012), Basque Country (Yurrebaso *et al.*, 2011), Austria (Dognaux *et al.*, 2011), Belgium (Berti *et al.*, 2010), Germany (Seider *et al.*, 2010), Hungary (Martín *et al.*, 2007), Italy (Berti *et al.*, 2010), North Italy (Cortellini *et al.*, 2011), Sweden (Albinsson *et al.*, 2011), U.K. (Tucker *et al.*, 2011a), Pol. (Reichert and Pawlowski, 2009), Poland (Hatzer-Grubwieser *et al.*, 2011)].

In this case, the comparison of our Portuguese sample ("Portugal") with other European samples revealed great homogeneity.

However, there were also some statistical differences observed, concerning this genetic marker, between Poland (Reichert and Pawlowski, 2009) and Sweden (Albinsson *et al.*, 2011) and Poland (Reichert and Pawlowski, 2009) and U.K (Tucker *et al.*, 2011a) (Figure 21). However, after applying the Bonferroni correction ($P < 0.05 / 5 = 0.01$), the deviations were considered statistically insignificant.

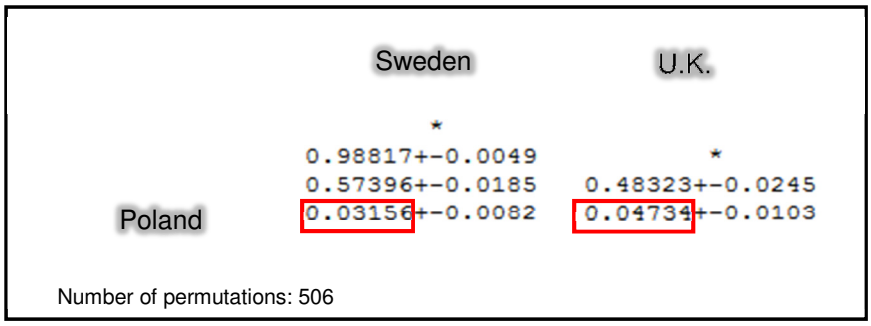


Figure 21 - Statistical deviations detected for the D10S1248 genetic marker between European samples.

D12S391

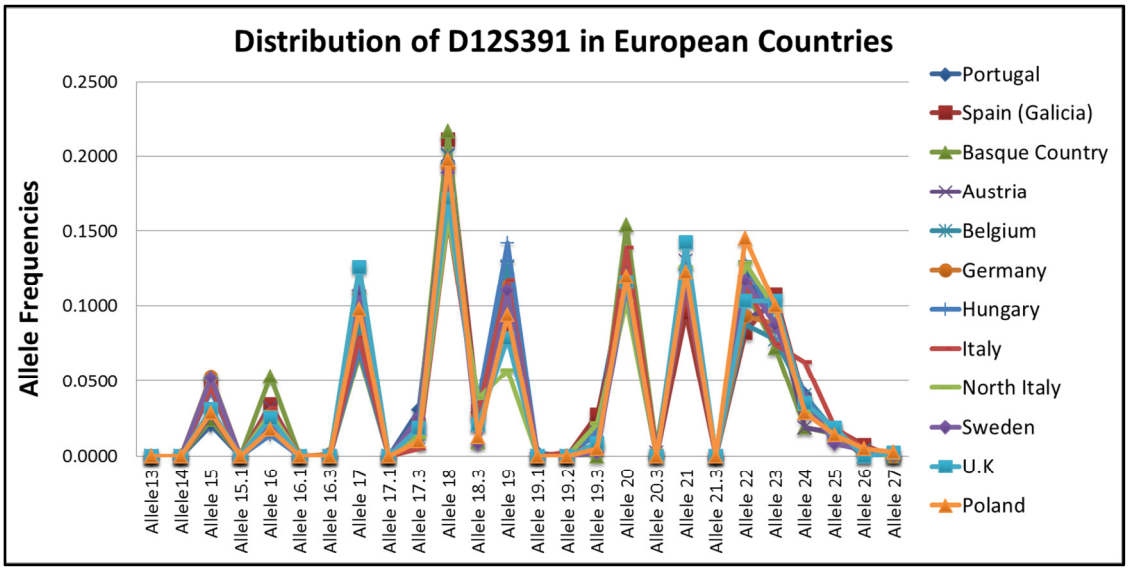


Figure 22 - Graphic representation of the allele frequency distribution for the D12S391 marker, in European samples.

Concerning the D12S391 allele distribution, in Figure 22, it is also possible to observe the homogeneity among European countries [Spain(Galicia) (Formoso *et al.*, 2012), Basque Country (Yurrebaso *et al.*, 2011), Austria (Dognaux *et al.*, 2011), Belgium (Berti *et al.*, 2010), Germany (Seider *et al.*, 2010), Hungary (Martín *et al.*, 2007), Italy (Berti *et al.*, 2010), North Italy (Cortellini *et al.*, 2011), Sweden (Albinsson *et al.*, 2011), U.K. (Tucker *et al.*, 2011a), Poland (Hatzer-Grubwieser *et al.*, 2011)].

For this locus, no statistically significant differences were observed between all European samples.

D22S1045

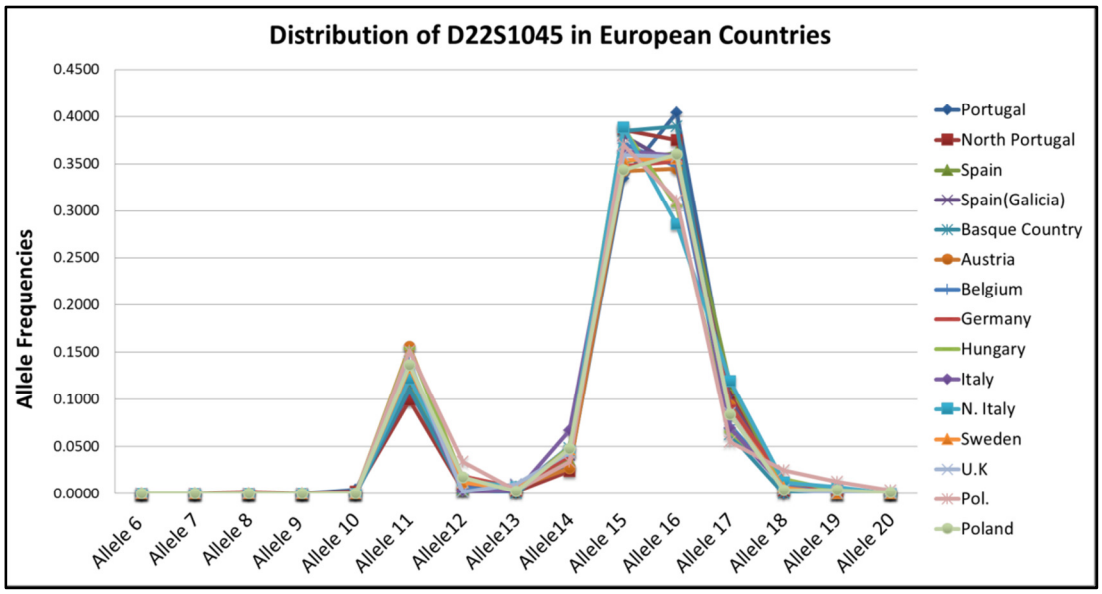


Figure 23 - Graphic representation of the allele frequency distribution for the D22S1045 marker, in European samples.

Relatively to the D22S1045 marker, the allele distribution between the European countries is represented in Figure 23 [North Portugal (Lagoa *et al*., 2008), Spain (Martín *et al*., 2007), Spain(Galicia) (Formoso *et al*., 2012), Basque Country (Yurrebaso *et al*., 2011), Austria (Dognaux *et al*., 2011), Belgium (Berti *et al*., 2010), Germany (Seider *et al*., 2010), Hungary (Martín *et al*., 2007), Italy (Berti *et al*., 2010), North Italy (Cortellini *et al*., 2011), Sweden (Albinsson *et al*., 2011), U.K. (Tucker *et al*., 2011a), Pol. (Reichert and Pawlowski, 2009), Poland (Hatzer-Grubwieser *et al*., 2011)]. Once more, this comparison between our Portuguese sample and other European samples revealed, apparently, no significant allele frequency differences.

However, some statistical differences were observed between our sample Portugal and Hungary (Molnár *et al*., 2011) and Portugal and North Italy (Cortellini *et al*., 2011) (Figure 24).

After applying the Bonferroni correction ($P < 0.05 / 5 = 0.01$), no deviations were considered statistically significant.

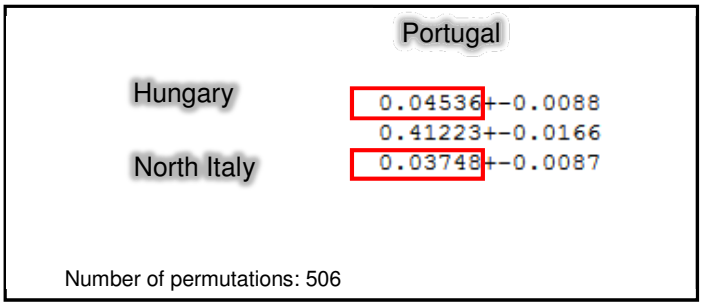


Figure 24 - Statistical deviations detected for the D22S1045 genetic marker between European samples.

Overall, our findings suggest that there are no significant allele frequency differences for the five new genetic markers between our Portuguese sample and the other European samples available in the literature.

Therefore, as expected from several autosomal STR population studies done throughout the years on major European samples, these five new markers show an overall homogeneous distribution. Moreover, since high values for forensic parameters were generally obtained, it may be admitted that these loci will improve significantly the discrimination power of IPATIMUP's internal database for application in kinship and genetic identification analyses.

4.2. Concordance Study

This kind of study allows the observation of any discordant genotype result for the same samples using different kits, with different performances and primer design. Detection of discrepancies between multiplex kits may be due to silent alleles (primer binding site polymorphisms) or the presence of microvariations outside the repeat motifs (Alves *et al.,* 2001, Alves *et al*., 2003, Amorim *et al*., 2004, Hill *et al*., 2007).

Since two of the kits used in this study share the same set of loci, with different primer sequences, it was assessed the possibility of any allelic drop-out or null alleles be present in the data set.

In this study, from almost 7200 alleles compared between the ESSplex Plus and the NGM systems (2 kits x 15 loci x 2 alleles/locus x 120 samples), full concordance between the typing results for the two kits was observed in 100% STR allele calls compared.

In the concordance process involving Identifiler Plus, Powerplex 16 HS and the samples genotyped by ESSplex Plus and NGM kits, full concordance was also obtained between these systems for the 8 STR markers (plus Amelogenin) they share.

4.3. Contamination Study

In order to guarantee reliable analyses, the potential for a possible contamination has to be evaluated for the multiplex system used in the laboratory routine. Thus, in the contamination study all blank controls amplified with the four different kits (ESSplex Plus, NGM, Identifiler Plus and Powerplex 16 HS), during the period involving the internal validation laboratory work, were run and noise levels evaluated. This not only allowed to verify good laboratory practice, but also to establish the analytical threshold (AT) to be used in IPATIMUP's laboratory casework, particularly important for low template DNA interpretation. The AT, expressed in RFUs, is the value where the observed peaks below it cannot be reliably distinguished from instrument noise (baseline signal) (Butler, 2011a). This threshold has a large impact on DNA fragment analysis and interpretation especially when dealing with degraded samples, samples containing low-levels of DNA and in complex mixtures. On one hand, if the AT is arbitrarily high, true signals will be incorrectly left unlabeled and will be lost during the analysis. On the other hand, an AT which is too low will not exclude considerable background noise from analysis, increasing the probability that randomly high baseline noise be incorrectly detected and labeled as true alleles (Bregu, 2011).

The contamination study for Identifiler Plus and Powerplex 16 HS was done due to the fact that these two systems have already been applied in routine casework. So, a comparison between a new option and the routine systems makes sense when the goal is to check which of the multiplex kits available may be more reliable or more sensitive. Achieving a common AT may also be useful, so that it can be always applied independently of the multiplex chosen.

For many years, the typical values applied by forensic laboratories for the ATs lied between 50-200 RFUs. Currently, since all of the new multiplex kits were recently designed for increased sensitivity, it is expected to observe a much lower and cleaner background noise. Therefore, the typical value applied nowadays for the AT in the forensic community, varies between 30 RFU – 50 RFU (Butler, 2011b, Butler, 2012, Coble, 2012, Sivak, 2011).

After evaluating all the blank controls studied in this work, it was decided that it was reasonable to establish the 30 RFU limit as the AT for all four multiplex kits. In all cases, the typical baseline level appeared around the 10 RFU threshold, with some noise peaks close to 20 RFU, and rarely few above this threshold (Figure 25 - 28). In low template DNA interpretation, it is important to maximize allele detection while at the same time minimize the false labeling of noise. The 30 RFU threshold seems conservative enough and sufficiently broad enough to accomplish this and allow standardization in STR allele interpretation.

ESSplex Plus

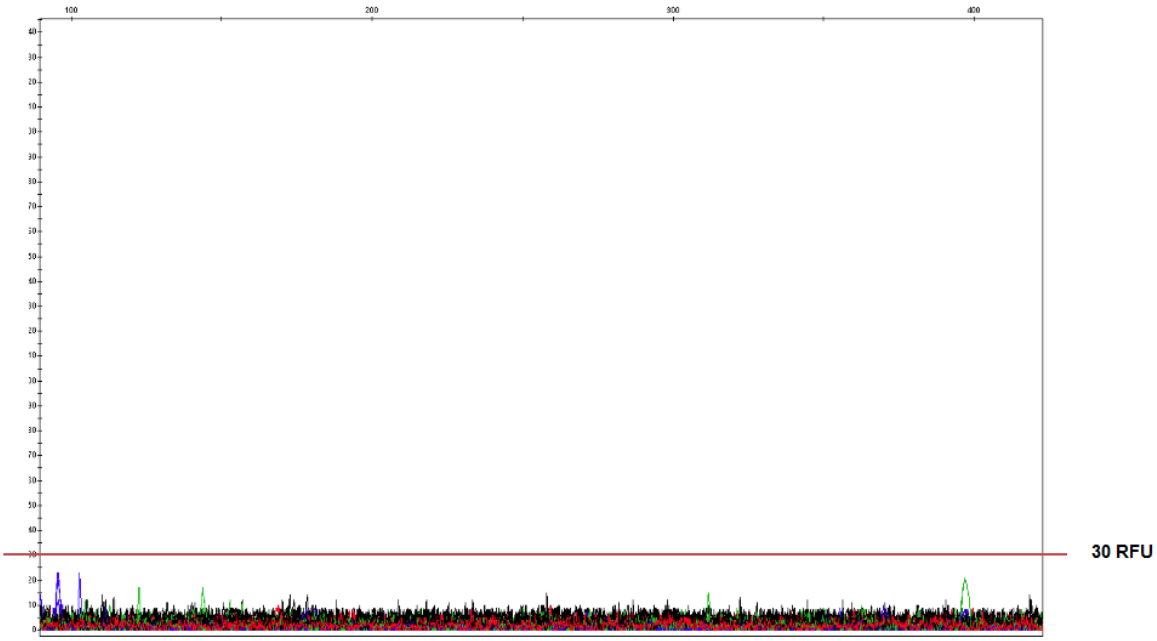


Figure 25 - Typical result of a blank control amplified with the ESSplex Plus system.

NGM

Figure 26 - Typical result of a blank control amplified with the NGM system.

Identifiler Plus

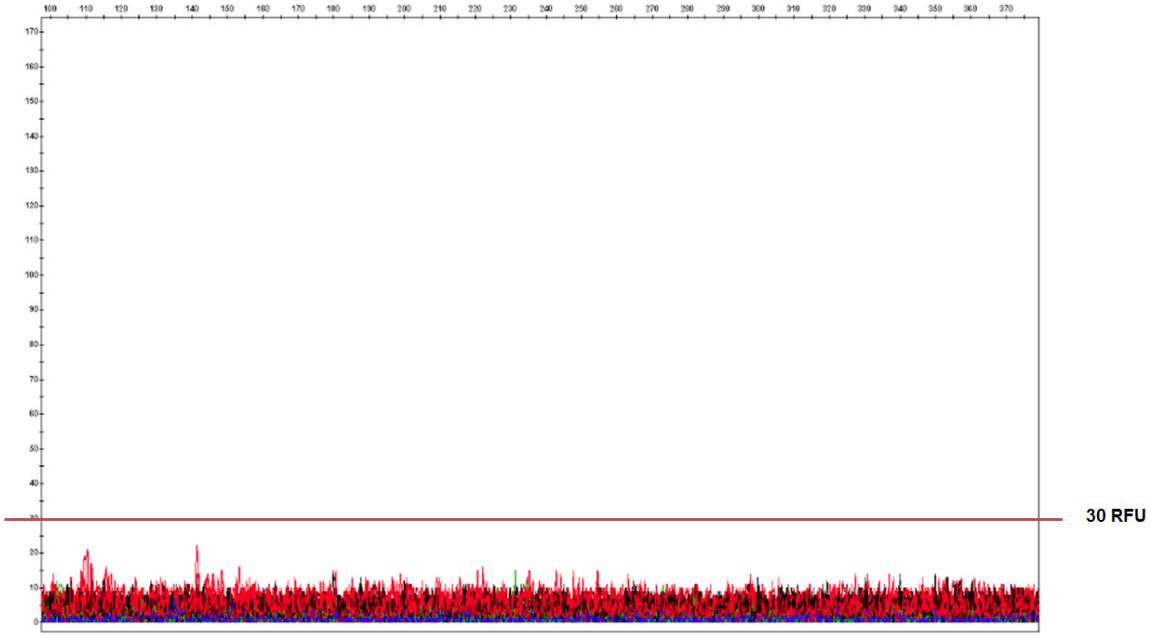


Figure 27 - Typical result of a blank control amplified with the Identifiler Plus system.

Powerplex 16 HS

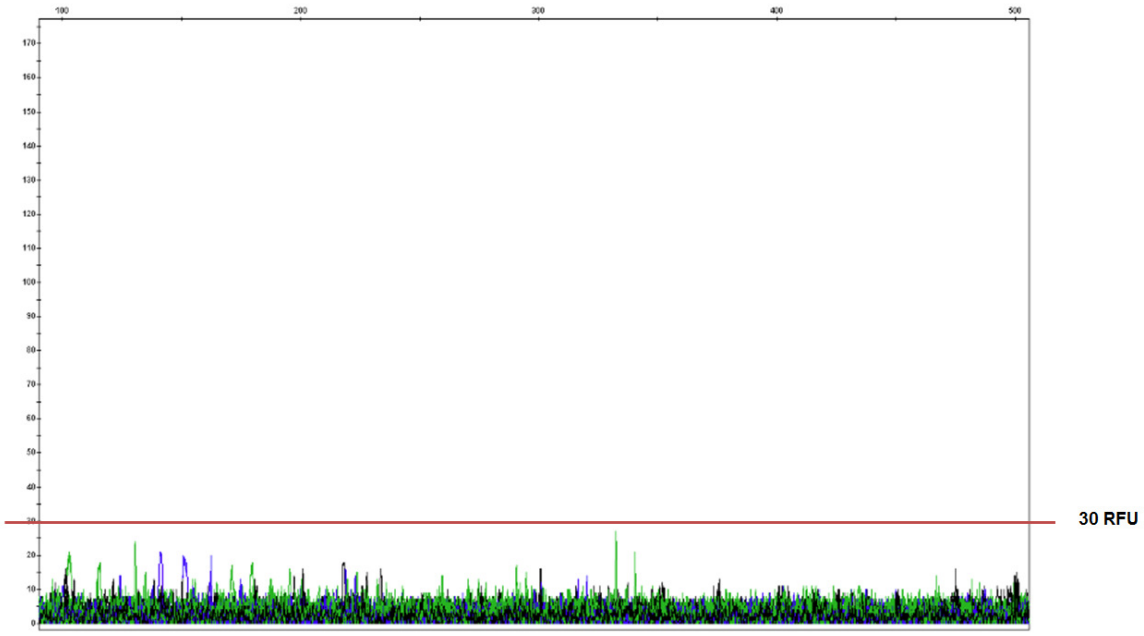


Figure 28 -Typical result of a blank control amplified with the Powerplex 16 HS system.

4.4. Sensitivity Study, Stochastic Effects and Artefacts

Before performing any other analysis involved in internal validation procedures, and since it is usually difficult to predict the amount of DNA after extraction, all DNA samples were quantified, using RT-PCR, by applying the recently introduced kit Investigator Quantiplex from Qiagen (Qiagen, 2011a).

In order to get a clear idea of the sensitivity of multiplex kits and since most stochastic effects are DNA input dependent, a robust quantification procedure is a fundamental tool for obtaining more precise results.

The kit chosen (Investigator Quantiplex, Qiagen) for the RT-PCR process has demonstrated high sensitivity and reproducibility in our study. It was used in IPATIMUP's laboratory for the first time during this study. The reaction is based on the amplification of a small fragment with 146 bp, being this region present on several autosomes of the human genome. Detection of amplification is performed using Scorpion primers and a novel, fast PCR chemistry. Scorpion primers are bifunctional molecules containing a PCR primer covalently linked to a probe. The fluorophore in this probe interacts with a quencher, also incorporated into the probe, which reduces

fluorescence. During PCR, when the probe binds to the PCR products, the fluorophore and quencher become separated. This leads to an increase in fluorescence in the reaction tube (Qiagen, 2011a). One of the advantages of this quantification kit is that it contains an internal control, 200bp, which is designed to be more sensitive to inhibitors than the human quantification target. The comparison of the CT value of the internal control system for DNA standards with the CT values of the internal control system for unknown samples may provide an indication of potential inhibition of the reaction in the unknown samples. This allows the detection of the presence of inhibitors in DNA samples, which in turn may compromise the multiplex STR PCR which follows (Qiagen, 2011a).

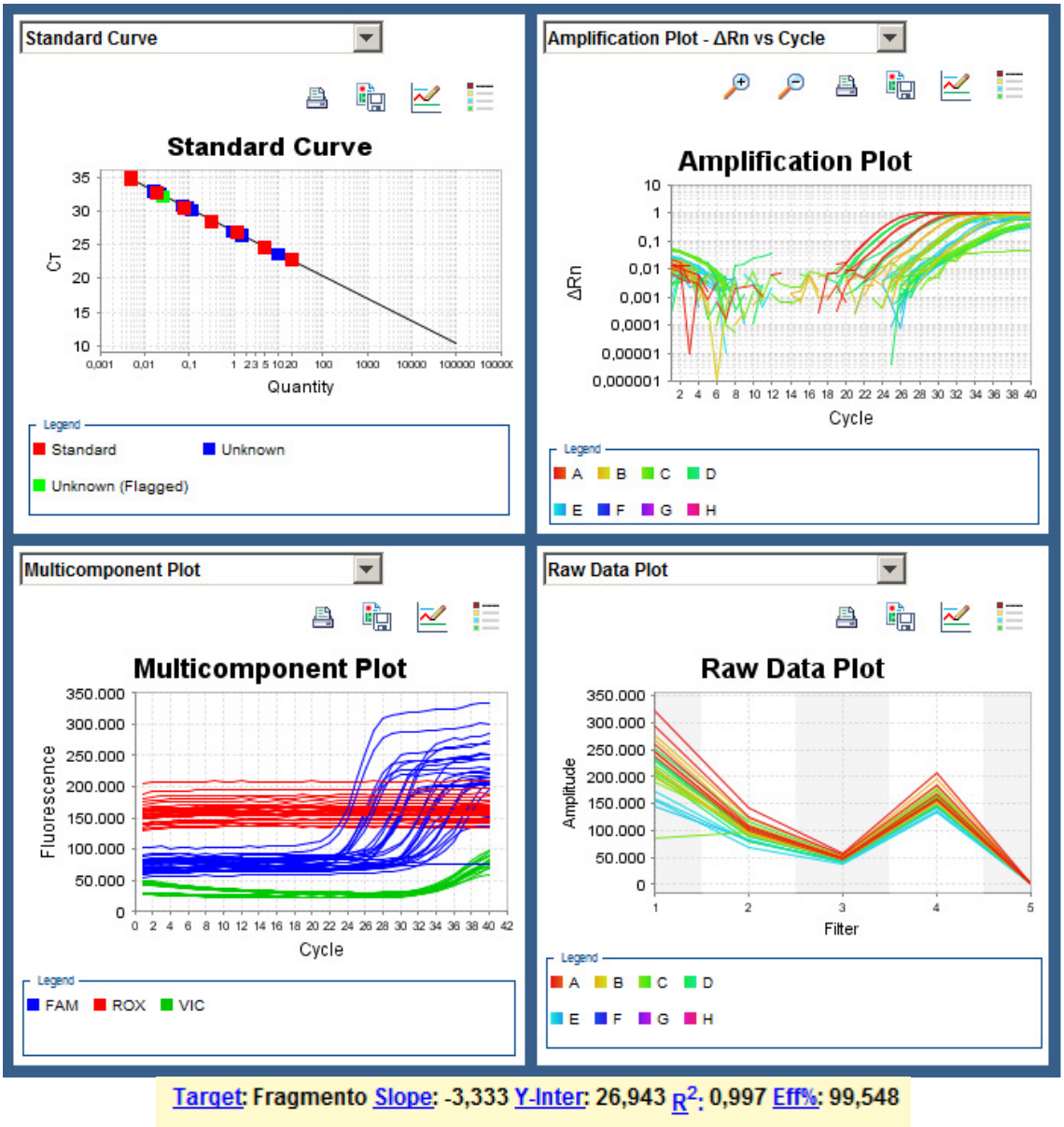


Figure 29 - Example of a graphic representation of the quantification results, concerning Standard Curve, Amplification Plot, Multicomponent plot and Raw Data Plot.

In Figure 29, the most important parameters of a standard curve are represented, as an example of the usual results that were obtained: the slope, the Y-intercept, the R^2 value and the efficiency. Relatively to the slope, it describes the PCR efficiency and typically ranges between -3.0 to -3.6. A value of -3.3 indicates 100% PCR efficiency, meaning that the number of copies of amplification product is doubled at each cycle (Qiagen, 2011a).

The R^2 value is a measure of the fit of the data points to the regressed line and usually the standard curve has an R^2 value ≥ 0.990. Moreover, the Y-intercept value is just an indication of the C_T (threshold cycle) value for a sample with Qty = 1(ng/µl) (Qiagen, 2011a).

Concerning the standard curve, it is expected that variability will occur in the quantification process of a same sample in different RT-PCR reactions. Therefore, the quality of the standard curve produced is important for obtaining reproducible and reliable results, and so caution must be taken to ensure that control DNAs for standard curve construction are well prepared. On the other hand, since guaranteeing equivalent standard curves in different RT-PCRs is almost impossible, it is fundamental to include in each reaction a control DNA sample with known DNA quantity. In this way, deviations between RT-PCR quantifications are more easily detectable.

4.4.1. Sensitivity

According to the ESSplex Plus Handbook (Qiagen, 2011b), the recommended input DNA for optimal results should be between 0.2 – 0.5 ng. Previous studies available through a developmental validation document, made by Qiagen (Qiagen, 2012), demonstrated that reliable results may be obtained with < 0.1 ng DNA. The aim of the analyses undertaken in this work was to check whether these recommendations held true for the type of samples routinely processed at IPATIMUP's laboratory.

DNA samples from different sources were serial diluted to obtain seven different concentrations (ranging from 0.5 ng/µl to 5 pg/µl), amplified and run according to the procedure already described. In Fig. 30 an example is shown of the results obtained from the ESSplex Plus kit with one of these serial dilutions.

Data was analysed by observing the overall performance throughout the decrease in DNA input. Stochastic effects such as allele imbalance, drop-out and drop-in, as well as artefacts such as stutter and pull-ups were measured, and genotypes were analysed by two approaches: visual and threshold. The "visual approach" is here understood as an EPG interpretation made by an experienced technician/researcher where no thresholds or reference values are taken into consideration, whilst the "threshold approach" is based on reference values, namely stutter and peak height ratios, an analytical threshold (AT) previously estimated and a stochastic threshold (ST), which are incorporated in the GeneMapper® ID Software v.3.2. analysis software (Applied Biosystems, 2005).

Note that the stochastic threshold delimits a peak height window where stochastic effects may be expected to occur. Above this peak height value, it is reasonable to assume that allelic drop-out of a sister allele of a heterozygote has not occurred at that locus, so single alleles above this value in single-source samples are assumed to be homozygous (Butler, 2012). According to (Butler, 2006c), this threshold is situated around 150 – 200 RFUs (Puch-Solis *et al*., 2011, Gill *et al*., 2009).

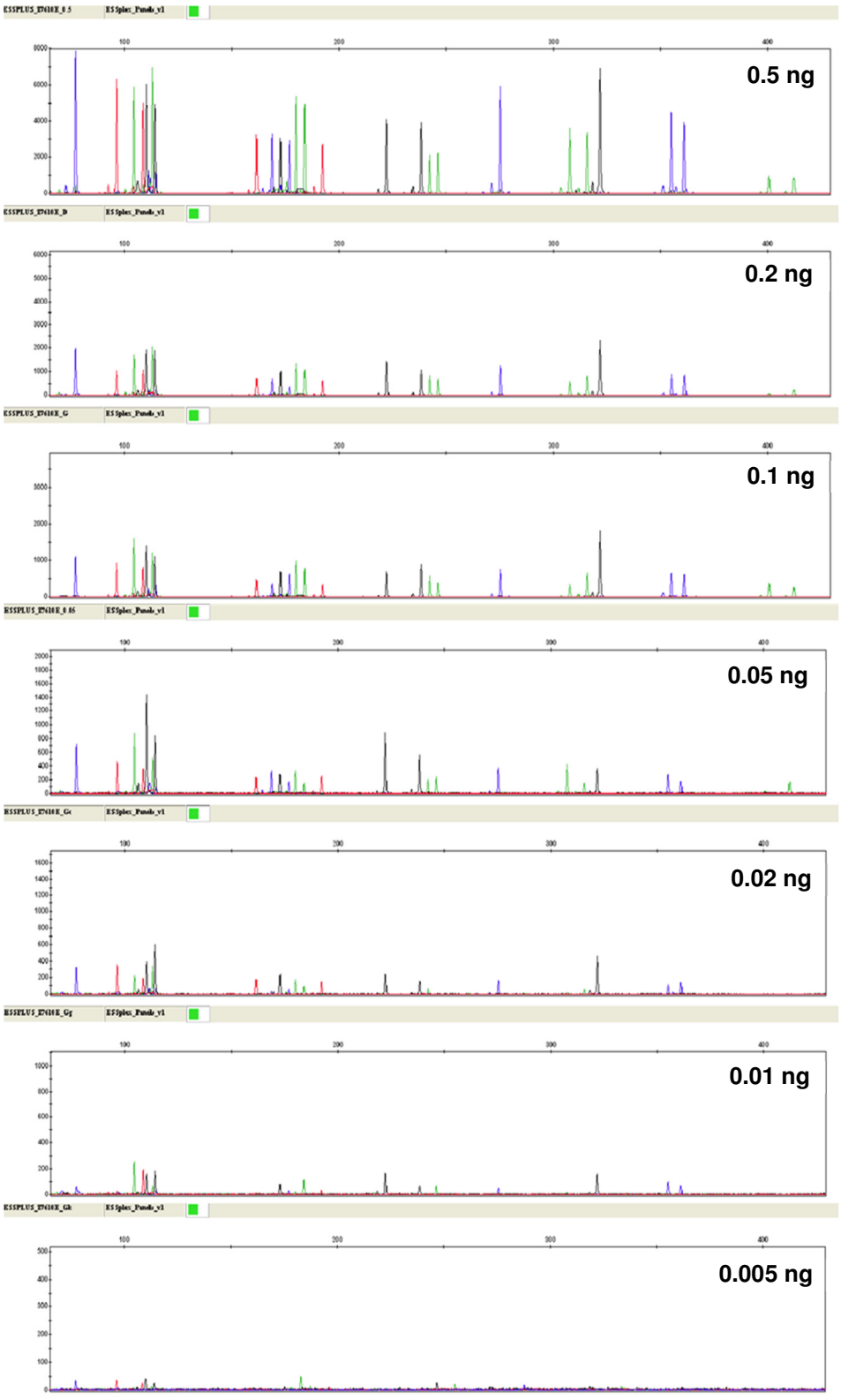


Figure 30 - Example of an electropherogram of a random sample analysed with ESSplex Plus kit. In this picture, the range of values experimented in the sensitivity test (from 0.5 ng/µl DNA to 0.005 ng/µl) is represented. Note that the y- axis scale was magnified for the smaller input amounts of DNA.

The absence of stochastic effects, together with the clear observation of balanced profiles and absence of pull-ups, determined the optimum amount of input DNA. This visual approach, applied on five different serial dilutions of samples from different sources, determined that full profiles for the ESSplex Plus kit are obtained down to 0.1 ng/ µl, since drop-out is observed for concentrations below 0.1 ng/ µl, which is consistent with what is indicated by the kit's reference values (from developmental studies).

Since at 1 ng/µl a slight pull-up effect was observed, the maximum recommended DNA concentration for reliable interpretation of genetic profiles was established to be 1 ng/µl. In this way, the optimum value estimated for input DNA was 0.5 ng/µl, where stochastic effects such as allele imbalance, drop-out and drop-in were absent, being in accordance with Qiagen recommended value (Qiagen, 2010, Qiagen, 2012).

4.4.2. PHR evaluation

After knowing the optimum DNA input, the peak height ratios (PHR) for all loci of the ESSplex Plus kit were estimated. Qiagen considers this value to be at 70% for all loci (Qiagen, 2012). However, due to individual compositions and inherent features of each genetic marker, it was considered relevant to estimate this parameter individually (Budowle *et al.*, 2009). Thus, heterozygous peak heights were measured; the average and standard deviation (SD) for each locus were obtained and finally applied the formula Average – SD to obtain the individual result for each one of the genetic markers (Figure 31).

Locus	Peak Height Ratio (%)
AMEL	84.3
TH01	75.1
D3S1358	72.3
VWA	70.8
D21S11	76.7
D16S539	80.1
D1S1656	76.0
D19S433	72.6
D8S1179	76.2
D2S1338	70.3
D10S1248	72.8
D22S1045	75.5
D12S391	73.1
FGA	74.8
D2S441	80.3
D18S51	75.1

Figure 31 - Peak height ratio (PHR) resulting from (average – SD), in percentage, for each ESSplex Plus locus.

The Amelogenin locus has no systematic bias that favours the amplification of Y and X alleles.

Since every locus has a PHR ≥ 70% (Figure 31), it can be admitted that above this value, two heterozygous alleles can be grouped as a possible genotype (Butler, 2012).

Usually, when the smaller of two peaks is less than 70% of the height of the larger peak at a locus, the disparity in peak heights is typically taken to be an indication that there is more than one contributor of template DNA to the sample. On the other hand, if the smaller of two peaks is equal to or greater than 70% of the height of the larger peak at a locus, that is consistent with the proposition that they may have come from a single contributor (Gilder *et al.*, 2011). This shows the relevance of this parameter in order to better evaluate cases where mixtures can be present (Gehrig *et al.*, 2011).

Although this threshold is in accordance with Qiagen's recommendations, it is important to remember that each locus has an individual behaviour, especially when using this ratio for mixtures analysis.

4.4.3. Stutter evaluation

In this study the stutter effect was evaluated concerning all loci of the ESSplex Plus Kit. The stutter ratio (%) was estimated per allele per locus according to (Budowle *et al.*, 2009), taking into account that it was not possible to cover the whole allelic range of each marker since each population has a particular allelic range.

Thus, a reference stutter value per locus was identified by taking the mean of each locus plus three times the SD (Applied Biosystems, 2011), in order to be useful especially for future mixture analysis.

The results obtained are represented across graphic distributions of the percentage of stutter peak detected against each locus allelic range, and are shown through Figures 32 to 46.

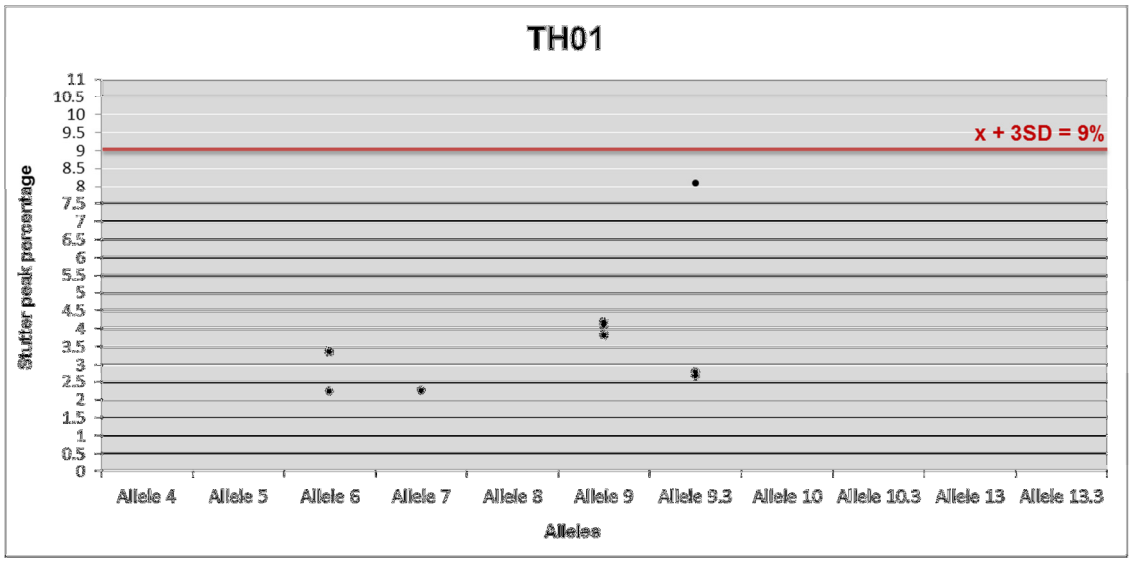


Figure 32 - Distribution of stutter values across the allelic range of the TH01 marker, in ESSplex Plus kit. Estimation of a a reference stutter value.

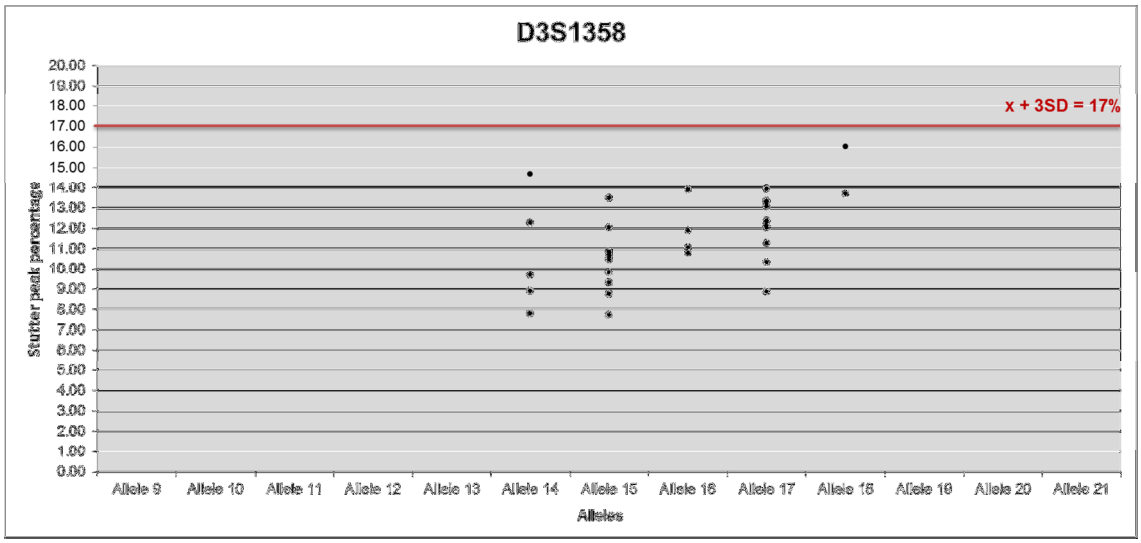


Figure 33 - Distribution of stutter values across the allelic range of the D3S1358 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

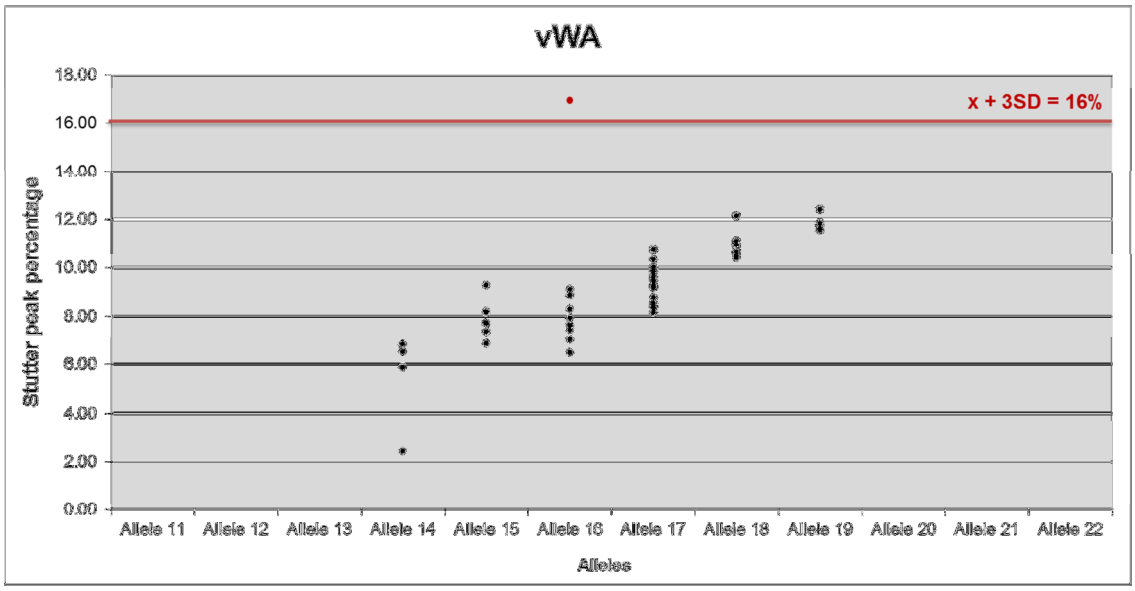


Figure 34 - Distribution of stutter values across the allelic range of the vWA marker, in ESSplex Plus kit. Estimation of a reference stutter value.

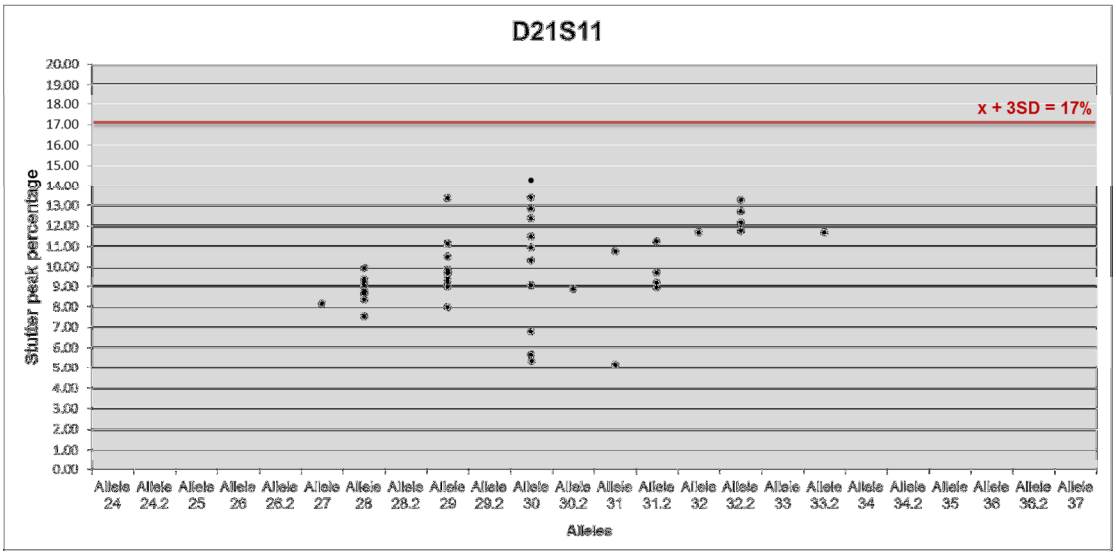


Figure 35 - Distribution of stutter values across the allelic range of the D21S11 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

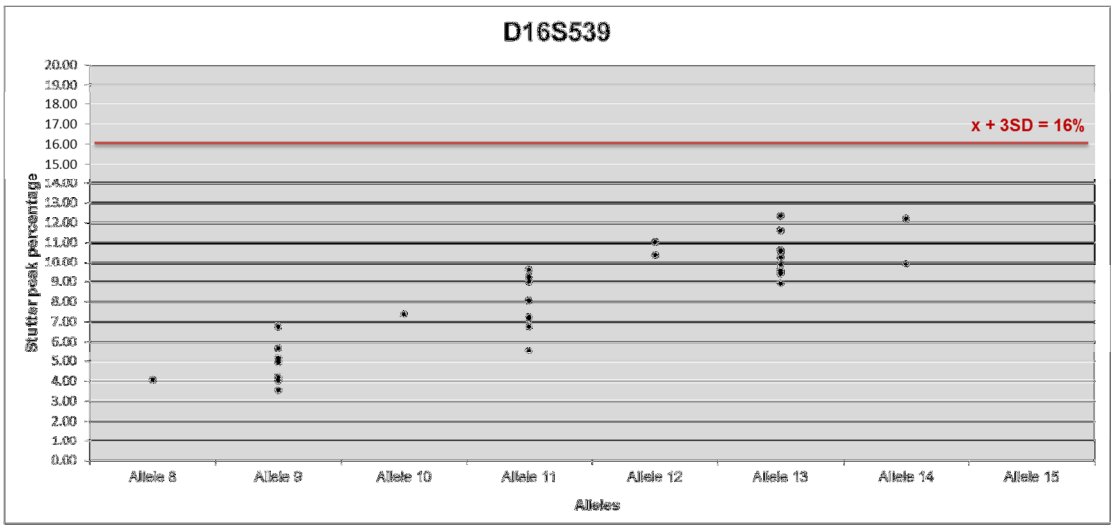


Figure 36 - Distribution of stutter values across the allelic range of the D16S539 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

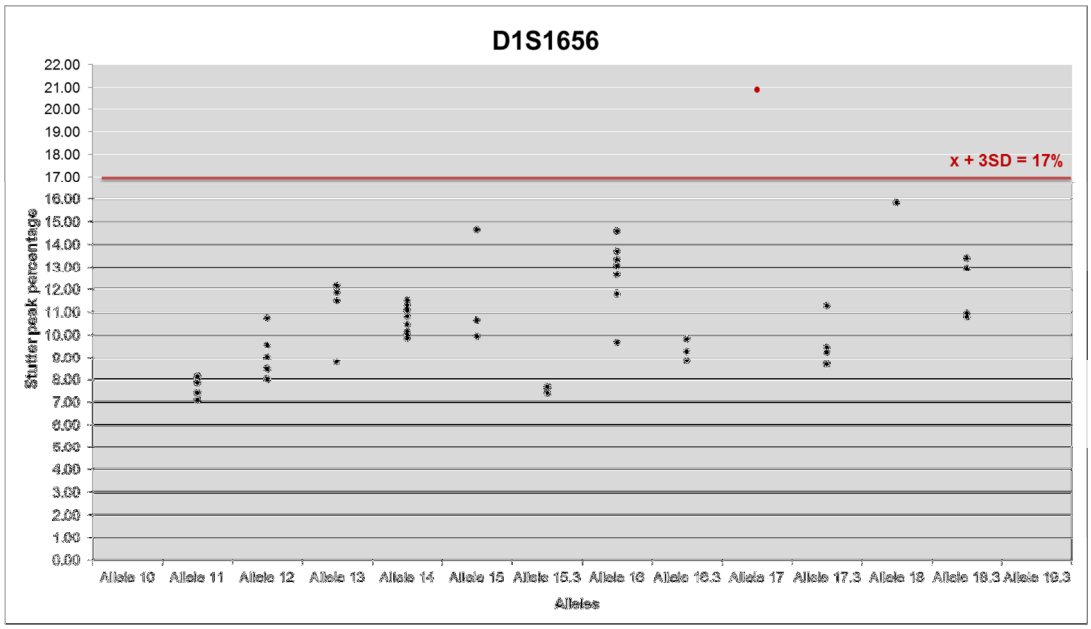


Figure 37 - Distribution of stutter values across the allelic range of the D1S1656 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

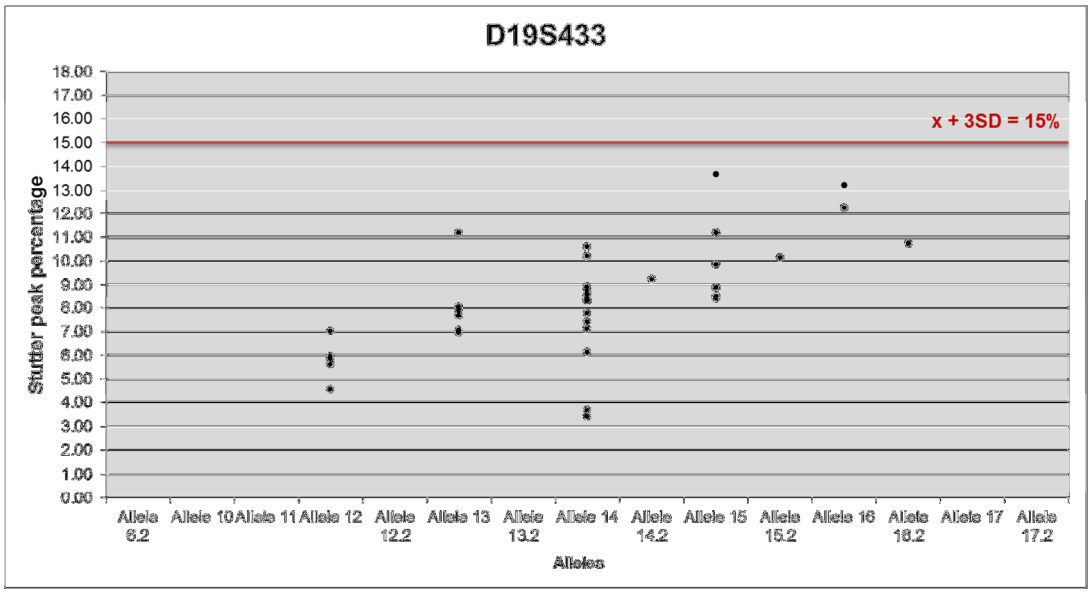


Figure 38 - Distribution of stutter values across the allelic range of the D19S433 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

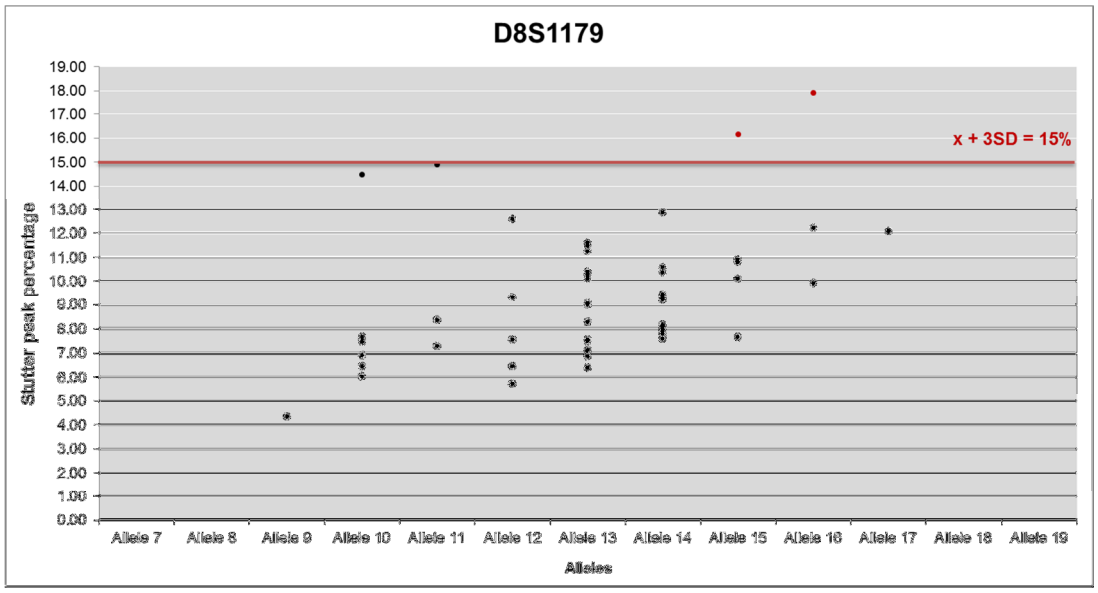


Figure 39 - Distribution of stutter values across the allelic range of the D8S1179 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

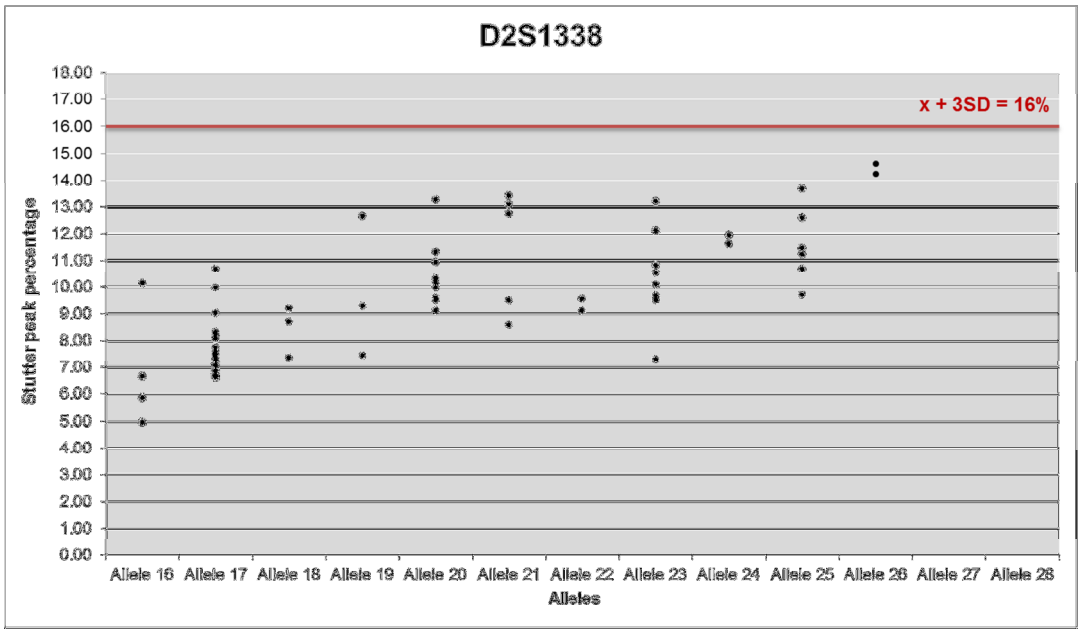


Figure 40 - Distribution of stutter values across the allelic range of the D2S1338 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

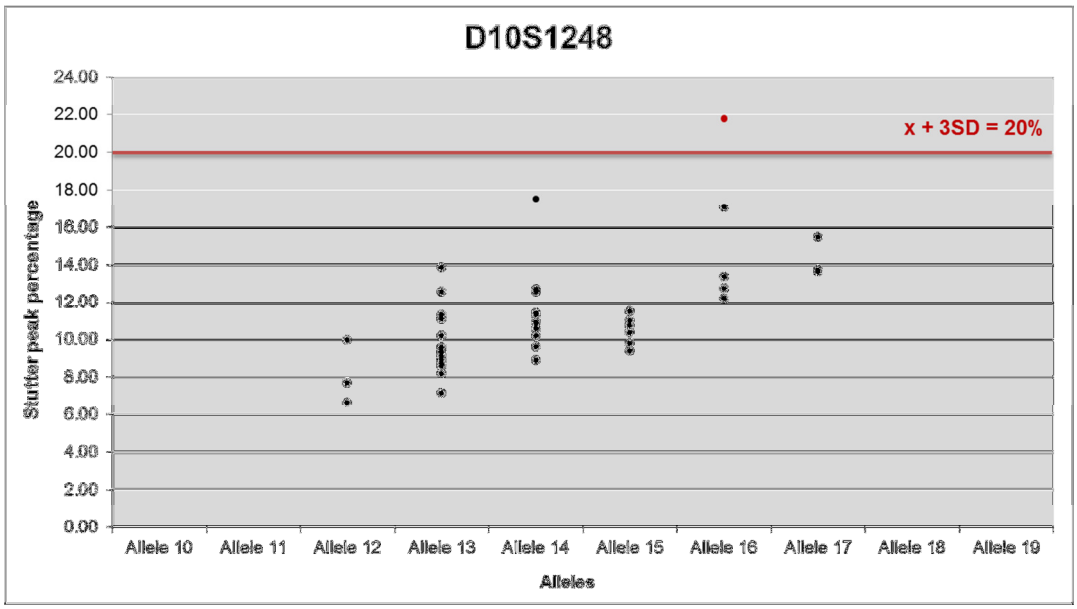


Figure 41 - Distribution of stutter values across the allelic range of the D10S1248 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

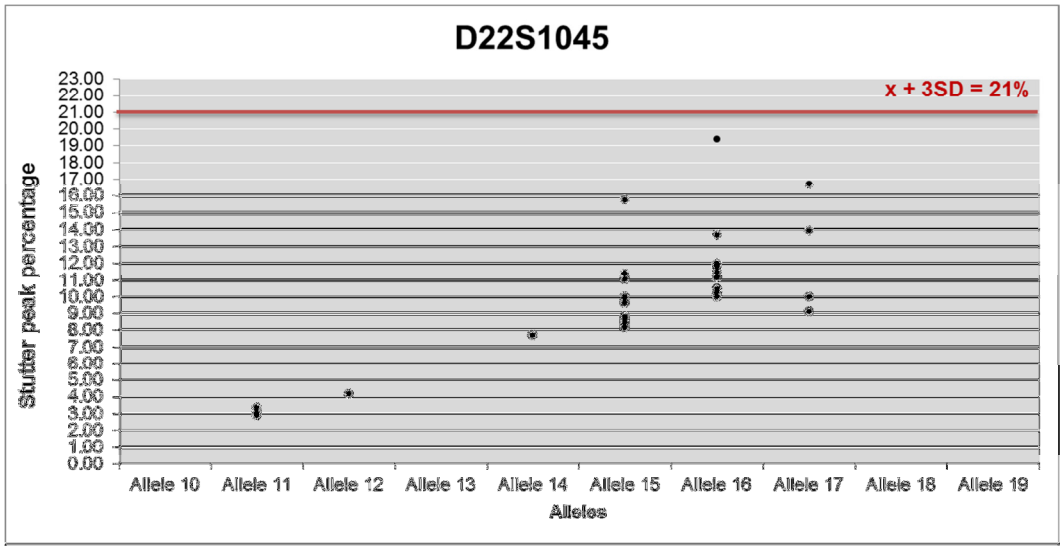


Figure 42 - Distribution of stutter values across the allelic range of the D22S1045 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

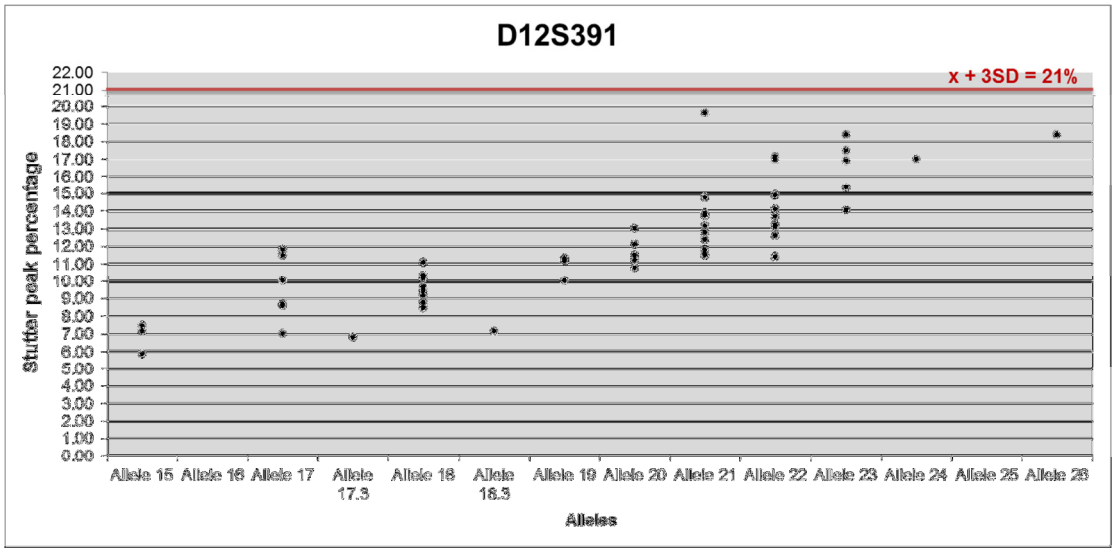


Figure 43 - Distribution of stutter values across the allelic range of the D12S391 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

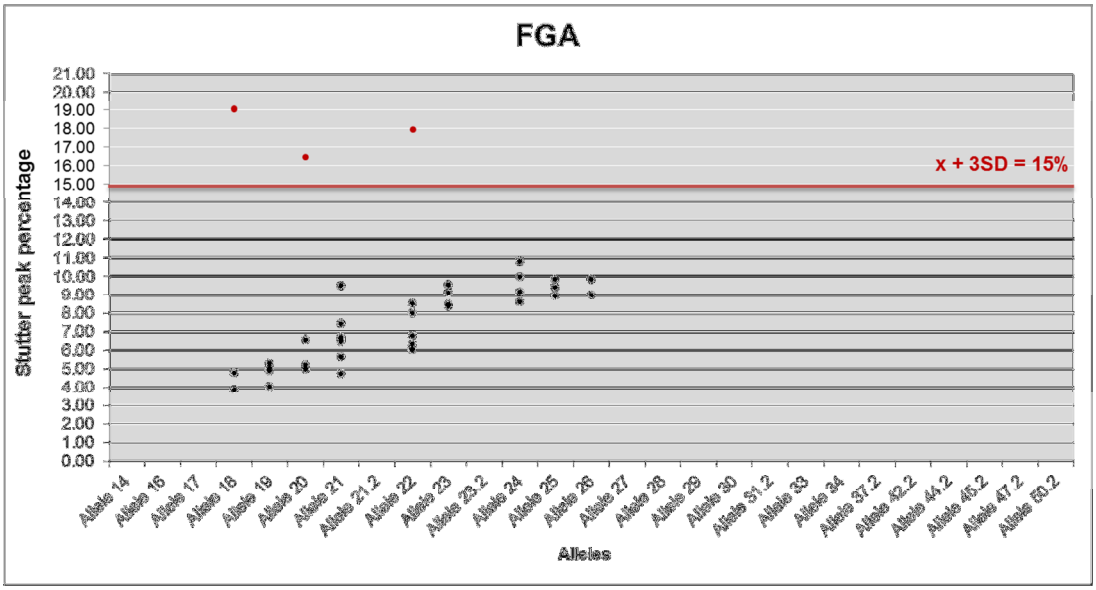


Figure 44 - Distribution of stutter values across the allelic range of the FGA marker, in ESSplex Plus kit. Estimation of a reference stutter value.

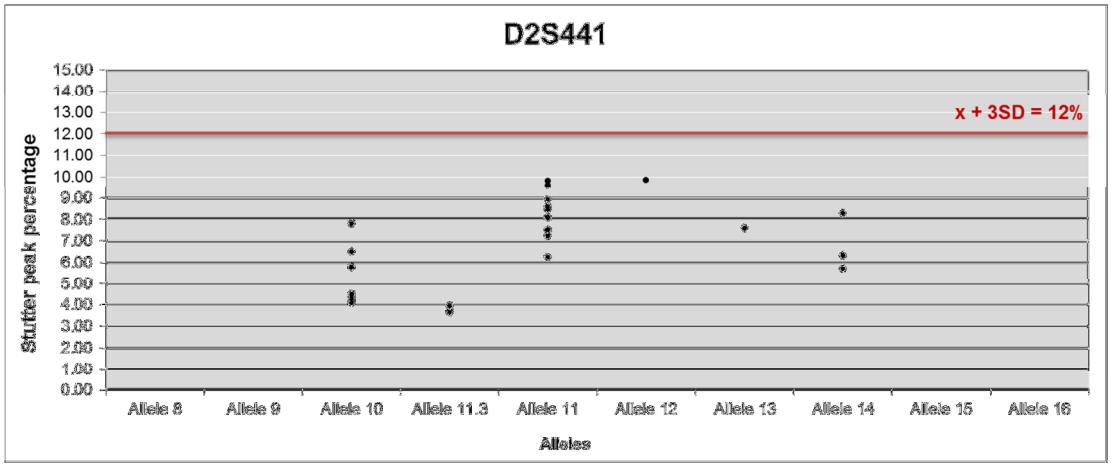


Figure 45 - Distribution of stutter values across the allelic range of the D2S441 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

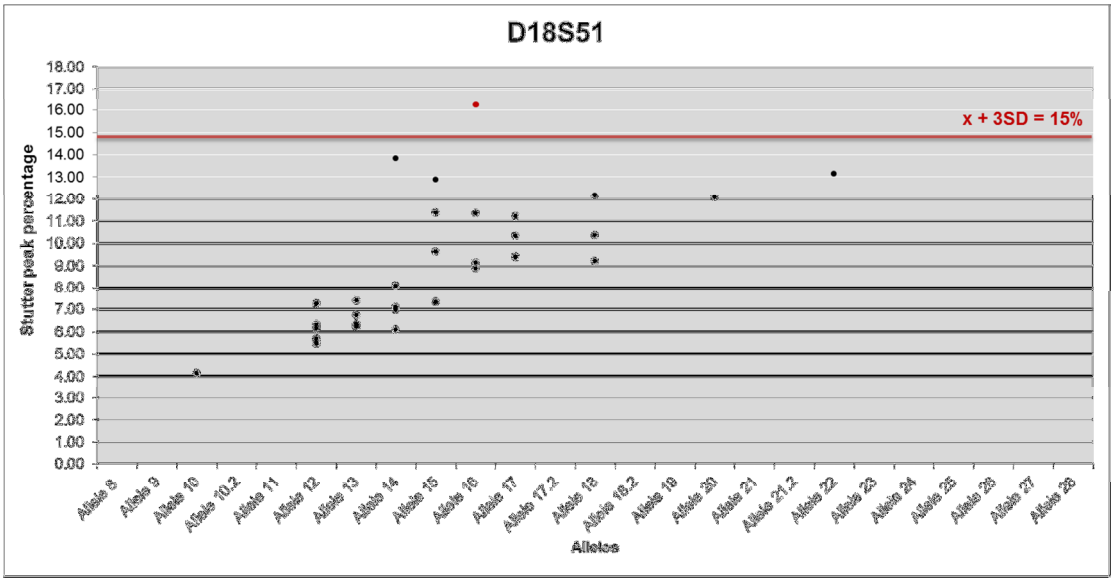


Figure 46 - Distribution of stutter values across the allelic range of the D18S51 marker, in ESSplex Plus kit. Estimation of a reference stutter value.

The range of stutter values (%) detected for this study, for the ESSplex Plus loci, was [9% - 21%], showing the heterogeneous behaviour inherent to each marker (Table 25).

Table 25 – Reference stutter values estimated for the ESSplex Plus loci, using the formula x + 3SD.

Locus	Stutter values (%)
AMEL	-
TH01	9
D3S1358	17
VWA	16
D21S11	17
D16S539	16
D1S1656	17
D19S433	15
D8S1179	15
D2S1338	16
D10S1248	20
D22S1045	21
D12S391	21
FGA	15
D2S441	12
D18S51	15

In this study it was also observed that the sequences and sizes of the STR alleles influence the stutter rates. Therefore, as expected, it was generally observed that the stutter effect increases with the increase of allele size for each locus (Brookes *et al.*, 2011).

Relatively to the repeat motif behaviour, in respect to their size, the D22S1045 locus (the only trinucleotide marker) has a high stutter value of 21%. This also confirms that the stutter effect is influenced by the size of the repeat motif, as is well known. The other markers, which are all tetranucleotide motifs, generally showed values of stutter lower than D22S1045.

The recommended stutter ratio values available from Qiagen (developmental studies) were compared with the values obtained in this internal validation study (Figure 47). Note that Qiagen uses the mean stutter ratio for each locus so, in order to compare with our study, the stutter ratios presented in Figure 47 correspond to the mean stutter ratio values.

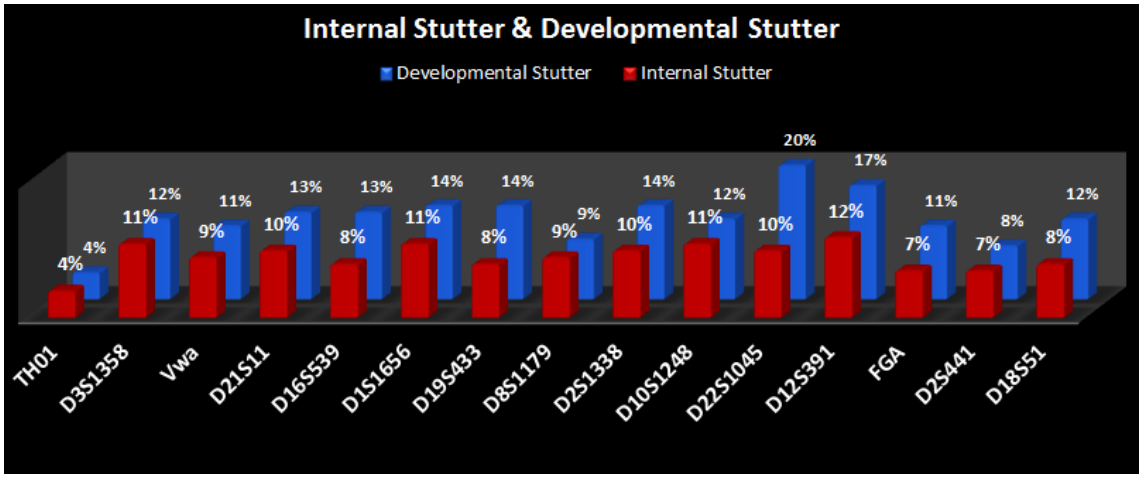


Figure 47 - Comparison between developmental mean stutter ratios and internal mean stutter ratios obtained in this study, for each locus of the ESSplex Plus kit.

The method employed in our study for locus stutter ratio determination (mean plus three times SD) allows for a more conservative approach especially when analysing mixture samples with GeneMapper ID v. 3.2. In Qiagen developmental studies, just the mean values are presented, without any statistical correction, being this sort of approach not recommended (Mulero *et al.*, 2008). Ideally, the best method would be to employ individual stutter ratios for each allele at each locus in a specific multiplex kit.

4.4.4. Sensitivity comparison between multiplex kits

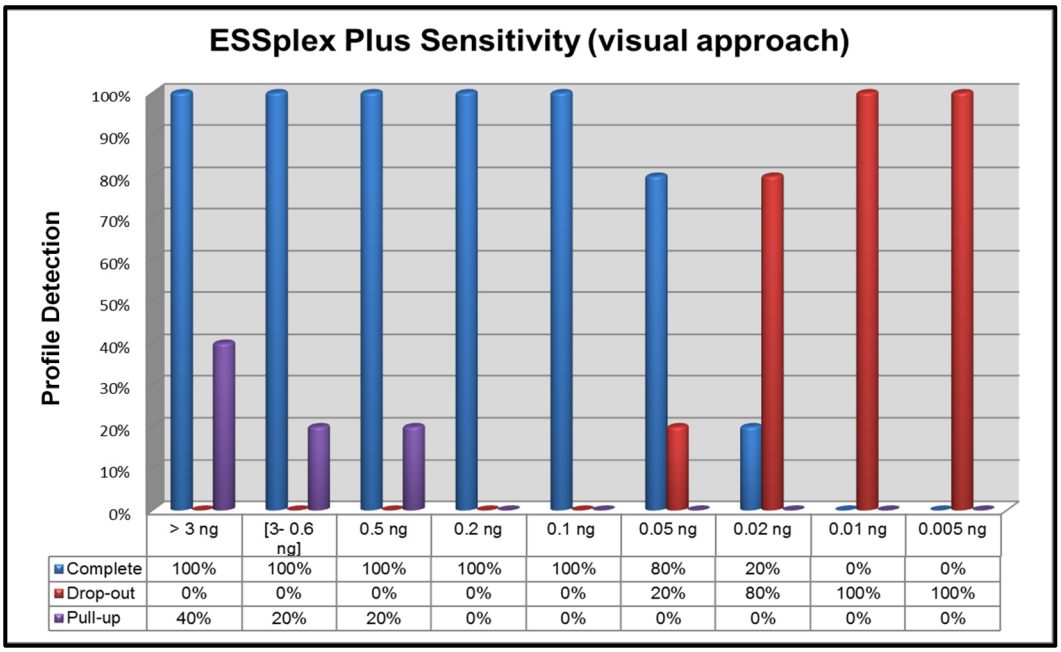
Since the recent next generation kits are said to have enhanced sensitivity, it seemed important to compare the sensitivity data obtained with the ESSplex Plus kit not only with the sensitivity results from the routine STR kits already employed in the laboratory (Identifiler Plus and Powerplex 16 HS), but also with the results obtained from direct ESSplex Plus competitors, like the NGM kit.

The serial dilutions of the samples used for the ESSplex Plus sensitivity study were also amplified with NGM, Identifiler Plus and Powerplex 16 HS kits. The results obtained are shown through Figures 48 to 55 and the comparison between methods and kits are discussed.

As mentioned earlier, the profiles obtained were analysed by two approaches: visual and threshold. As a reminder, the threshold approach is based on reference values,

namely stutter and peak height ratios previously estimated; the same analytical threshold (30 RFU) and stochastic threshold (150 RFU) were applied for all kits in the analysis of the genotypes. Note that at IPATIMUP's laboratory the ST has already been established as 150 RFU and seemed appropriate to apply this value in this work.

The analysis from both perspectives for the ESSplex Plus kit, (Figure 48 and Figure 49), revealed that the threshold approach is the most conservative, due to the more restrictive values involved (30 RFU for AT, 150 RFU for ST , the previously estimated values of stutter and PHR). In this approach, the drop-out effect emerged earlier, at 0.1 ng/µl (Figure 49), compared with the visual approach, at 0.05 ng/µl (Figure 48).

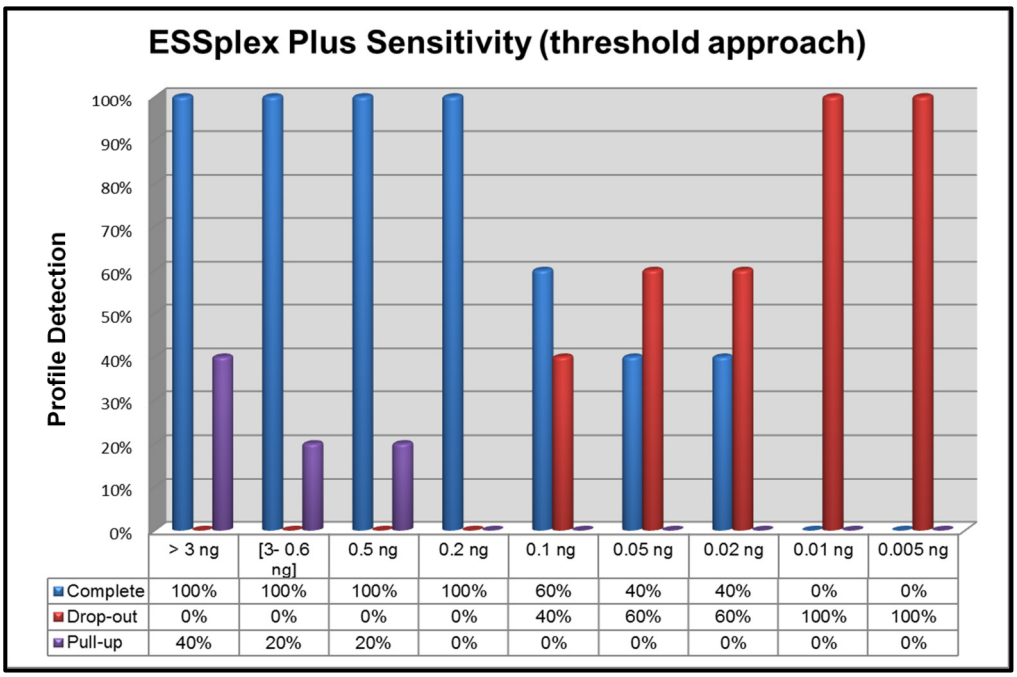


	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	100%	100%	80%	20%	0%	0%
Drop-out	0%	0%	0%	0%	0%	20%	80%	100%	100%
Pull-up	40%	20%	20%	0%	0%	0%	0%	0%	0%

Figure 48 - Sensitivity results of the ESSplex Plus Kit, concerning visual approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

Concerning the other parameters, the pull-up effect is considerably detected at values equal or above 2 ng/µl, where the difficulty in results interpretation may be greater. Nevertheless, this effect was also detected in samples with 0.5 ng/µl and, although this could constitute an exception, this isolated event has to be taken into account when analysing routine casework (Figure 48 and Figure 49). The drop-in effect was only detected at DNA input levels < 0.02 ng/µl.

The ESSplex Plus kit allowed the detection of complete profiles until 0.1 ng/µl using the visual approach (Figure 48) and 0.2 ng/µl using the threshold approach (Figure 49).

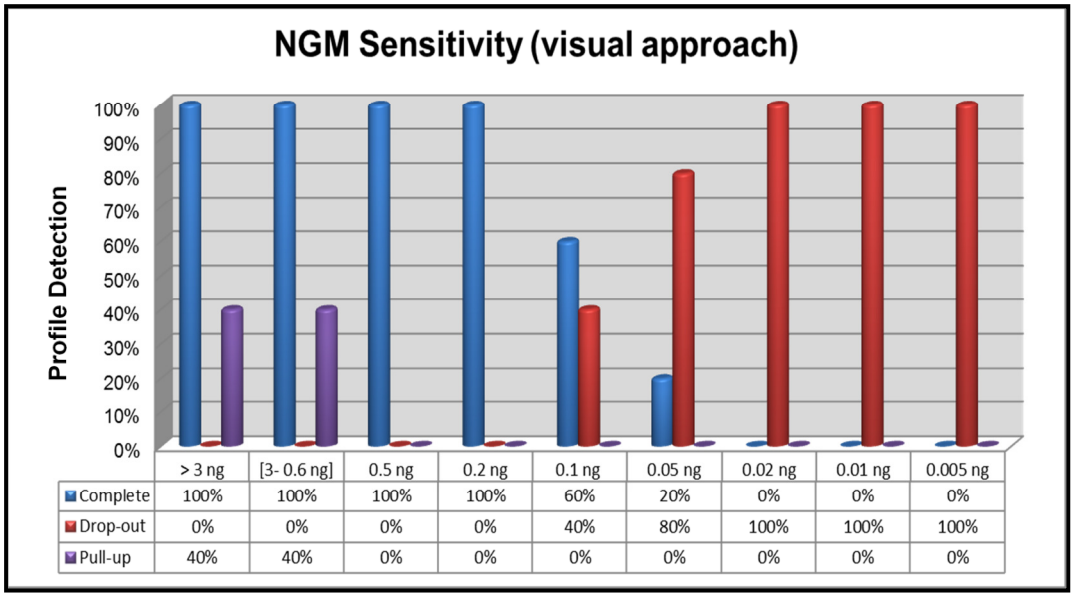


	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	100%	60%	40%	40%	0%	0%
Drop-out	0%	0%	0%	0%	40%	60%	60%	100%	100%
Pull-up	40%	20%	20%	0%	0%	0%	0%	0%	0%

Figure 49 - Sensitivity results of the ESSplex Plus Kit, concerning threshold approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

With the threshold approach, it is clear that nearly half of the profile information is lost at 0.1 ng/ µl, much sooner than in the visual approach.

Concerning the NGM kit, both approaches revealed similar results for each parameter studied (Figure 50 and Figure 51).

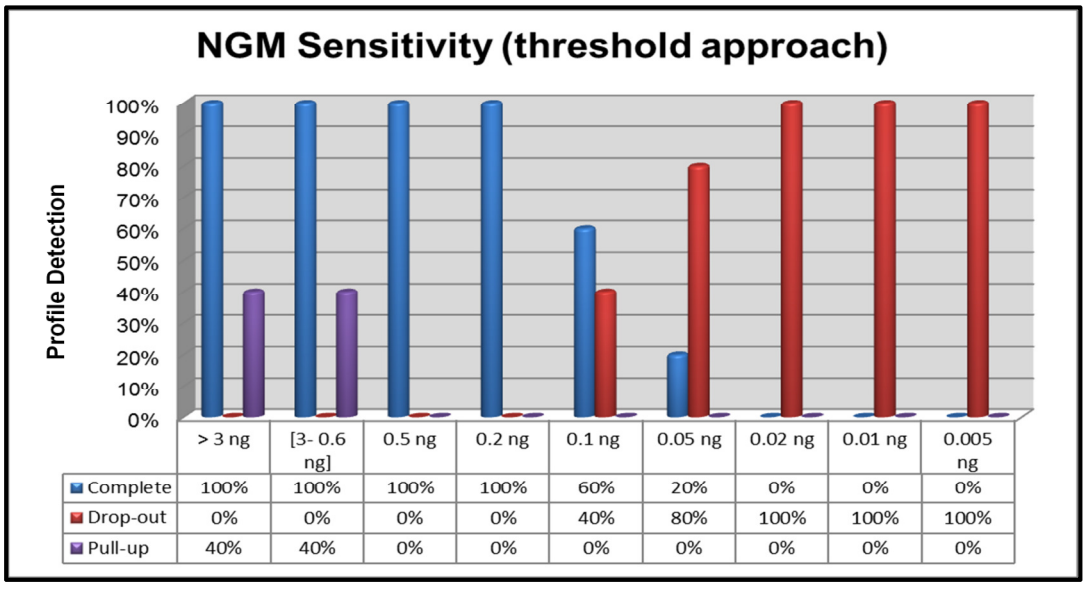


	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	100%	60%	20%	0%	0%	0%
Drop-out	0%	0%	0%	0%	40%	80%	100%	100%	100%
Pull-up	40%	40%	0%	0%	0%	0%	0%	0%	0%

Figure 50 - Sensitivity results of the NGM Kit, concerning visual approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

Nevertheless, this system revealed a premature detection of allele drop-out (at 0.1 ng/µl), which can indicate a worse performance compared to ESSplex Plus. Thus, complete profiles were only guaranteed until 0.2 ng/µl.

The pull-up effect appeared at values above 2 ng/µl. Relatively to the drop-in effect, it was only detected at 0.01 ng/µl of DNA.

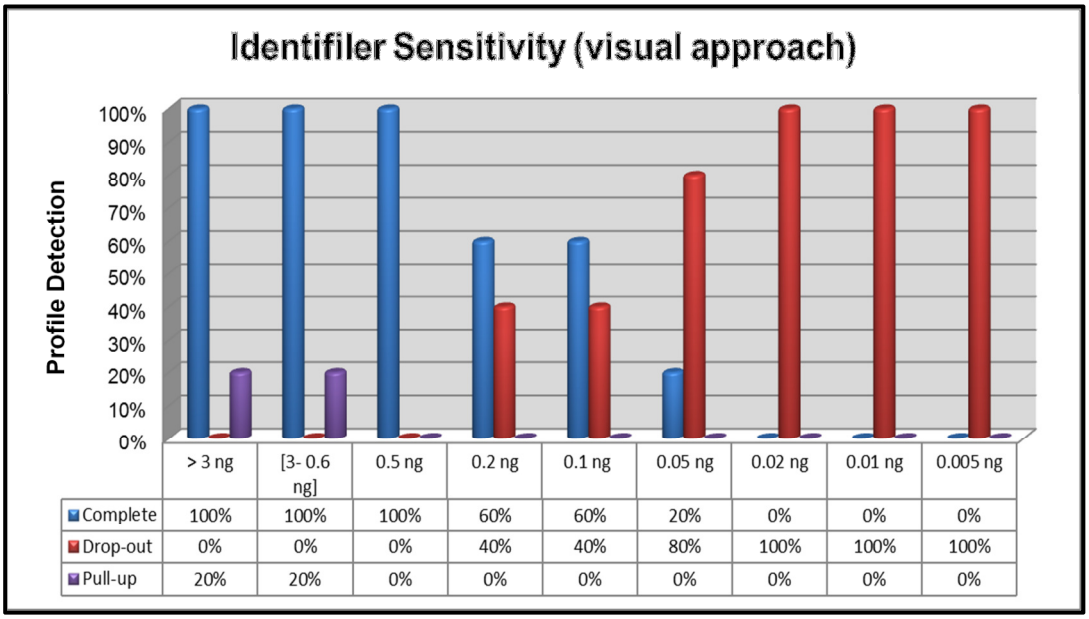


	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	100%	60%	20%	0%	0%	0%
Drop-out	0%	0%	0%	0%	40%	80%	100%	100%	100%
Pull-up	40%	40%	0%	0%	0%	0%	0%	0%	0%

Figure 51 - Sensitivity results of the NGM Kit, concerning threshold approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

Concerning the Identifiler Plus kit, the drop-out effect was detected even earlier (0.2 ng/µl) using both methods (Figure 52 and Figure 53), allowing for complete profiles to be obtained only after 0.5 ng/µl of input DNA.

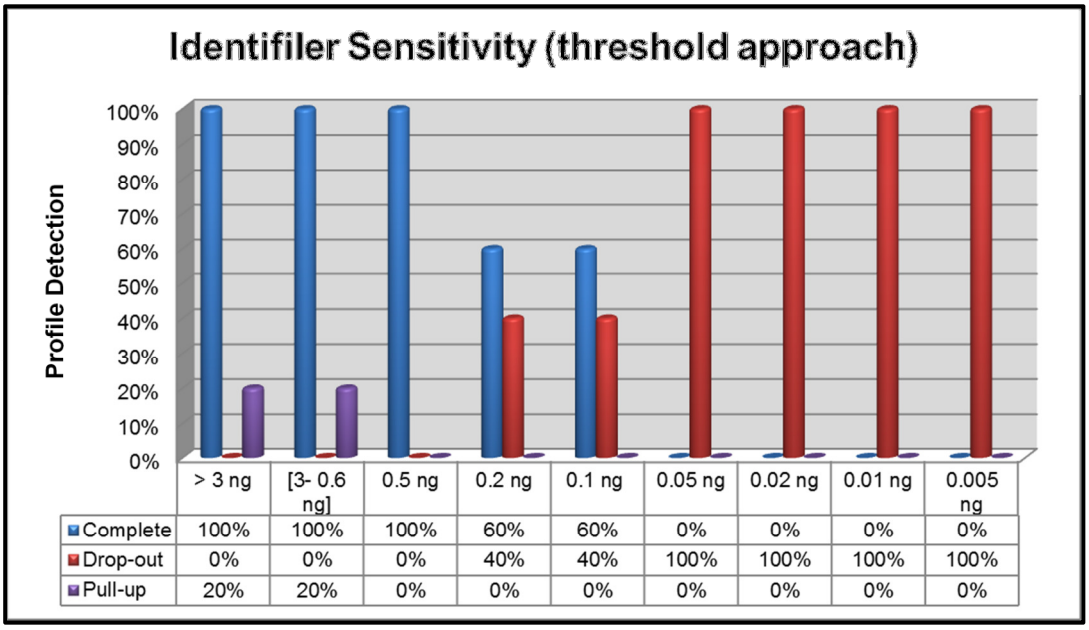
The pull-up effect was only detectable for values equal or above 2 ng/µl (Figure 52 and Figure 53) and drop-in was just present at 0.01 ng/µl of input DNA.



	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	60%	60%	20%	0%	0%	0%
Drop-out	0%	0%	0%	40%	40%	80%	100%	100%	100%
Pull-up	20%	20%	0%	0%	0%	0%	0%	0%	0%

Figure 52 - Sensitivity results of the Identifiler Kit, concerning visual approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

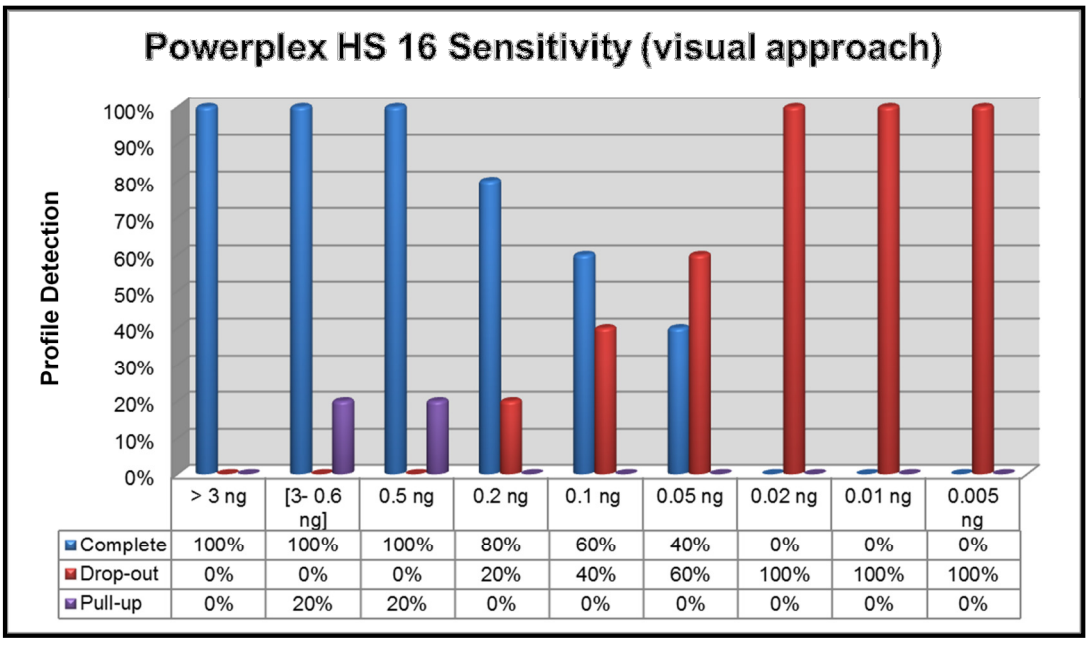
With Identifiler Plus, both perspectives showed similar performances. However, it was clearly visible the lower sensitivity of this system when compared with ESSplex Plus and NGM kits.



	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	60%	60%	0%	0%	0%	0%
Drop-out	0%	0%	0%	40%	40%	100%	100%	100%	100%
Pull-up	20%	20%	0%	0%	0%	0%	0%	0%	0%

Figure 53 - Sensitivity results of the Identifiler Kit, concerning threshold approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

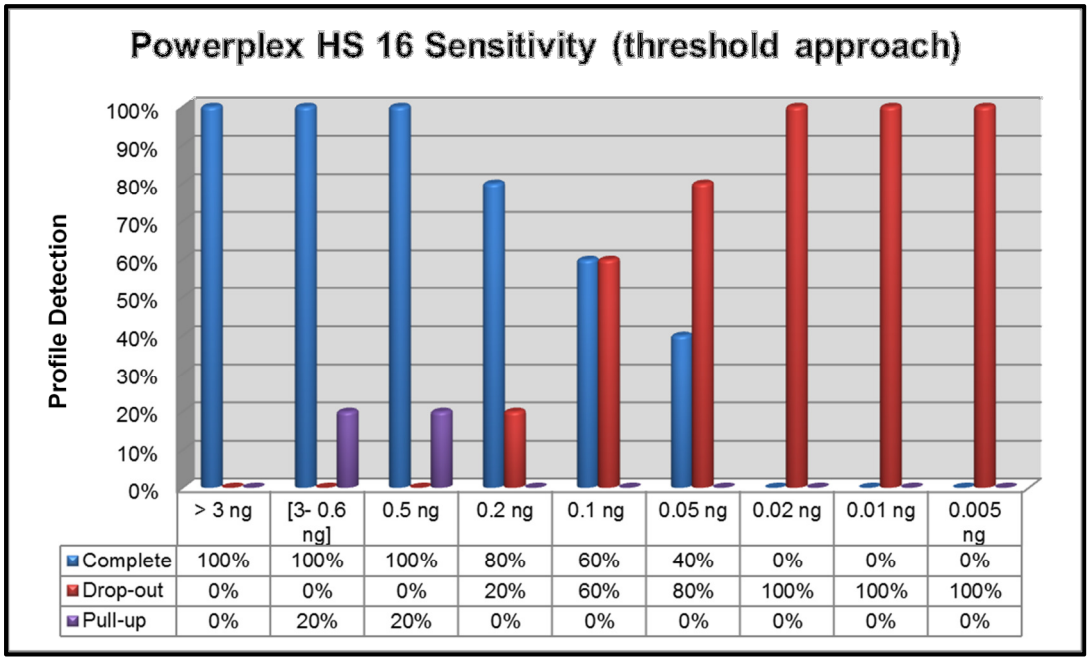
Finally, when the Powerplex HS 16 system was evaluated, the drop-out effect was detected at 0.2 ng/µl (Figure 54 and Figure 55) using both perspectives.



	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	80%	60%	40%	0%	0%	0%
Drop-out	0%	0%	0%	20%	40%	60%	100%	100%	100%
Pull-up	0%	20%	20%	0%	0%	0%	0%	0%	0%

Figure 54 - Sensitivity results of the Powerplex HS 16 Kit, concerning visual approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

The pull-up effect was detected for values greater than 0.5 ng/µl inclusive, although it was not observed above 3 ng/µl in the samples analysed. Drop-in was observed at 0.01 ng/µl of input DNA, with an isolated event at 0.1 ng/µl.



	> 3 ng	[3- 0.6 ng]	0.5 ng	0.2 ng	0.1 ng	0.05 ng	0.02 ng	0.01 ng	0.005 ng
Complete	100%	100%	100%	80%	60%	40%	0%	0%	0%
Drop-out	0%	0%	0%	20%	60%	80%	100%	100%	100%
Pull-up	0%	20%	20%	0%	0%	0%	0%	0%	0%

Figure 55 - Sensitivity results of the Powerplex HS 16 Kit, concerning threshold approach. Parameters such as complete profile, drop-out and pull-up were evaluated.

Overall, the drop-out phenomenon showed particular patterns. In a total of 20 drop-out events detected in the set of the 5 samples for the 4 multiplex systems, the D2S1338 marker was present in 11 events (55%), which is expected since this marker has the largest amplicons of the whole set. Also Powerplex 16 HS system had, essentially, three markers (Penta E, Penta D, and CSF1PO) as the first loci to suffer drop-out. In all cases, there is clear correlation between higher fragment size and more prone to allele drop-out.

In summary, after analysing the four multiplexes, the ESSplex Plus and the NGM kits showed better results and more sensitivity when compared with Identifiler Plus and Powerplex 16 HS, commonly used in routine casework. Thus, ESSplex Plus and NGM kits seem to be the selected systems when a more sensitive and reliable analysis is needed, concerning, for instance, cases of degraded and low copy number samples.

Moreover, through the serial dilutions performed with different samples sources (whole blood, blood stains, buccal swabs), at optimum concentrations of input DNA, it was possible to verify the reproducibility of the ESSplex Plus Kit, in the achievement of the same genetic profile across several PCR reactions of the same sample (obtained through different biological materials and different means of sample collection) and also in the achievement of reproducible balanced profiles.

4.5. Mixture Study

The increase in sensitivity has a consequent effect on mixture analyses, namely on the detection of minor mixture components. In these experiments, the ability of detecting the minor component was studied for the ESSplex Plus kit by determining the lowest mixture ratio at which the full profile of the minor component can be identified as well as determining at which mixture ratio it is possible to detect a mixture profile, according to the parameters used for this evaluation (more than two peaks per locus and peak height imbalance).

The results obtained by mixing samples from two individuals (male and female) in different proportions were evaluated taking into account the peak height ratio of 70%, the individual loci values of stutter and the AT (30 RFU) previously determined and incorporated in the GeneMapper ID analysis software (Figure 56 and Figure 57).

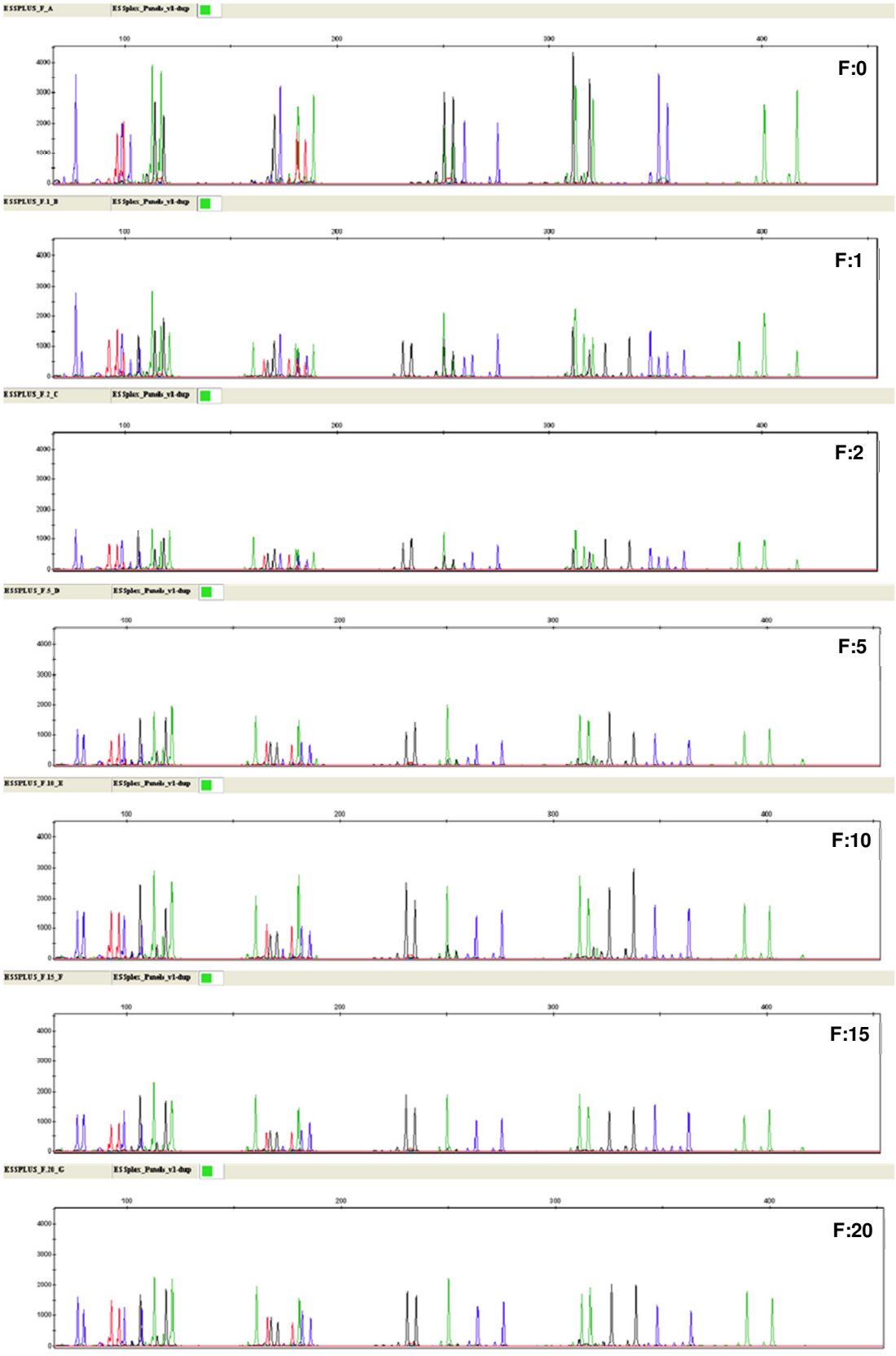


Figure 56 - Electropherogram of the experiment where increasing amounts of male DNA were mixed with constant female DNA in the following ratios: F:0, F:1, F:2; F:5, F:10, F:15 and F:20.

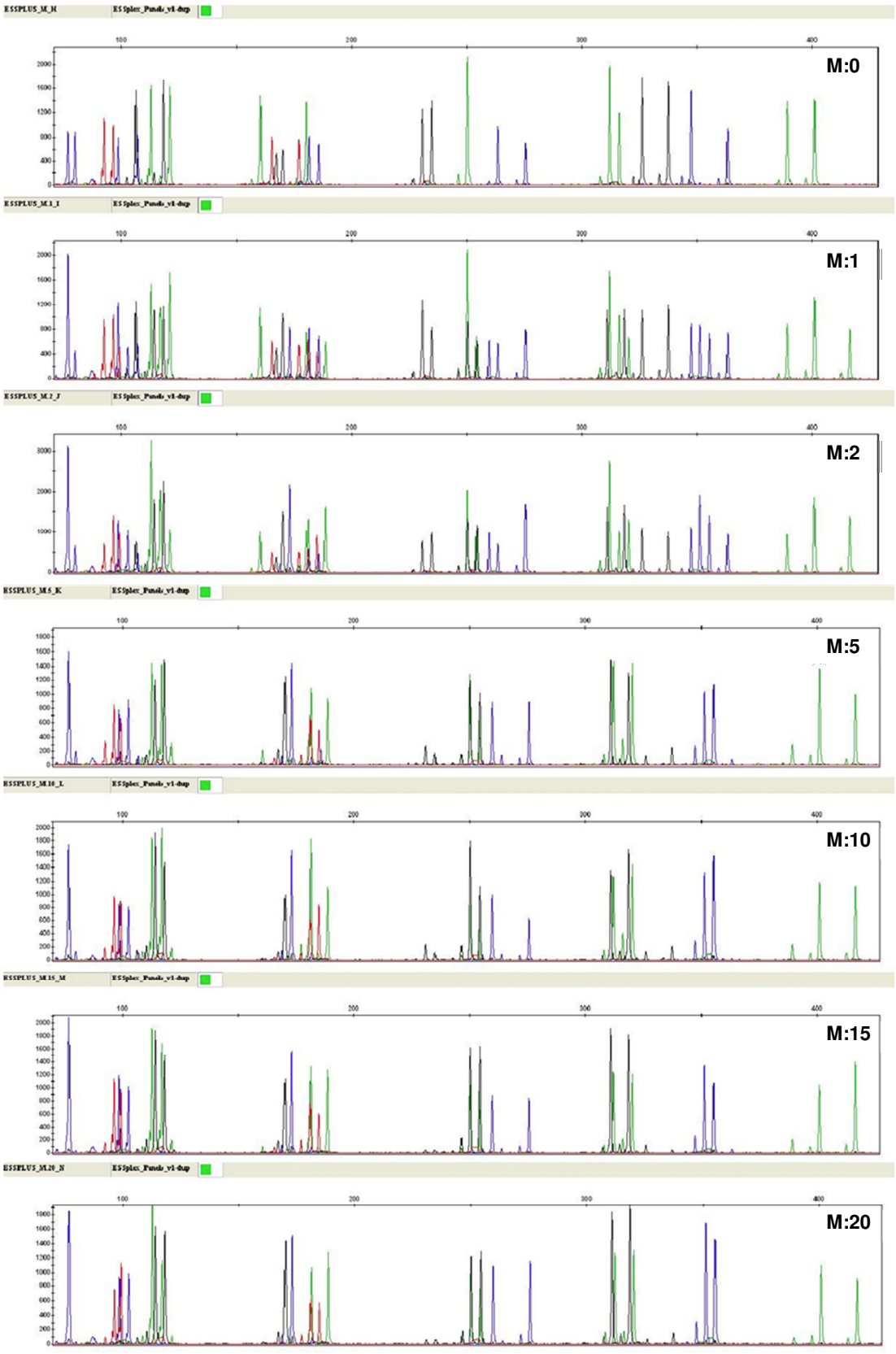


Figure 57 - Electropherogram of the experiment where increasing amounts of female DNA were mixed with constant male DNA in the following ratios: M:0, M:1, M:2; M:5, M:10, M:15 and M:20.

Concerning the analysis of mixtures ratios of increasing male DNA to constant female DNA, the analysis software determined a full profile of the minor component (female) at the 1:5 ratio. The analysis of the mixture ratios of increasing female DNA to constant male DNA revealed that a full profile of the minor component (male) was possible until the 1:2 ratio. In Figure 58, the electropherogram of the mixtures from 1:5 to 1:10 are shown for the blue fluorochrome to illustrate the loss (allele not called) of the minor contributor full profile. In the same way, concerning Figure 59, the electropherogram represents the mixtures from 1:2 to 1:5, for the yellow fluorochrome, illustrating also the loss (allele not called) of the minor contributor full profile.

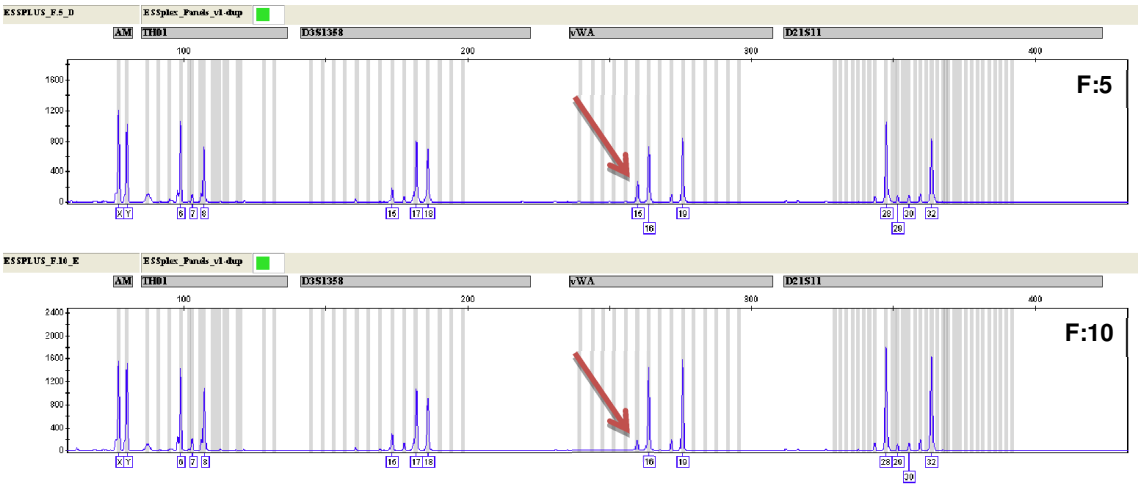


Figure 58 - Electropherogram of the mixtures from 1:5 to 1:10 are shown for the blue fluorochrome to illustrate the loss of the minor contributor full profile. The red arrow shows the allele not called by the Analysis Software.

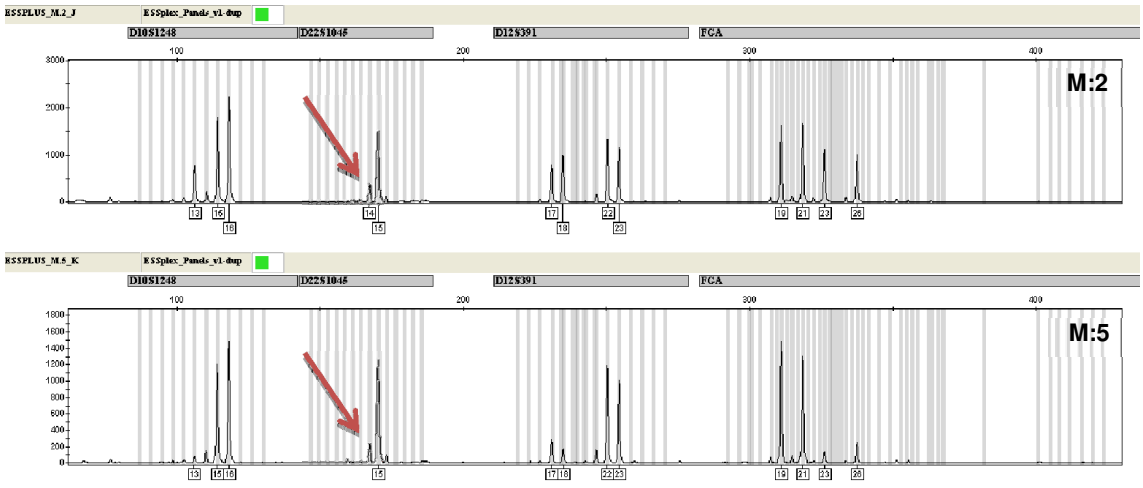


Figure 59 - Electropherogram of the mixtures from 1:2 to 1:5 are shown for the yellow fluorochrome to illustrate the loss of the minor contributor full profile. The red arrow shows the allele not called by the Analysis Software.

The ability of detecting a mixture profile by the analysis software is summarized in Table 26.

Table 26 - Mixture study results concerning two different analysis: female DNA constant with an increase of male DNA and male DNA constant with an increase of female DNA. Y (Yes) means a positive detection of mixture and N (No) an absence of mixture event. The red letters represent specific and isolated events.

	Female Constant						Male Constant					
	F:1	F:2	F:5	F:10	F:15	F:20	M:1	M:2	M:5	M:10	M:15	M:20
AMEL	Y	Y	N	N	N	N	Y	Y	Y	Y	Y	N
TH01	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
D3S1358	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N
VWA	Y	Y	Y	N	N	N	Y	Y	Y	Y	Y	Y
D21S11	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	N
D16S539	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
D1S1656	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
D19S433	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N
D8S1179	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N
D2S1338	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
D10S1248	Y	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y
D22S1045	Y	N	N	N	N	N	Y	Y	N	N	N	N
D12S391	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
FGA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
D2S441	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
D18S51	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

By evaluating all loci together, it is possible to detect clear mixture profiles at ratios of 1:20 (Table 26), for female or male constant DNA, according with the established parameters for mixture detection (more than two peaks per locus and peak height imbalance) and using the GeneMapper ID's analysis parameters.

As can be seen in Figure 58 and Figure 59, it is clear that some extra peaks are present although the software does not call them as alleles, because they are either at stutter positions or below the 30 RFU threshold. This emphasizes that mixture interpretation, at levels where major and minor contributors are very disproportionate, an application of a fixed stutter ratio is too conservative and valuable information will be lost, since alleles from the minor contributor will not be called. This study emphasizes that it is important to follow the current guidelines in mixture interpretation, stating that in mixtures with low level of a minor contributor, the alleles from the minor contributor may be in stutter positions of the major contributor, and should not be disregarded (Gill *et al.*, 2006a, Moretti *et al.*, 2001).

The locus by locus independent evaluation approach showed some isolated events between specific mixture ratios, which are depicted in Table 26 as red letter Y. In some cases, for example, the loss of the minor contributor detection at 1:15 ratio was

recovered again at 1:20. This may be due to the fact that PCR has different efficiencies and also that electrophoresis conditions vary considerably, namely injection efficiency.

This mixture study also allowed to better understand the relative ratio of DNA contributions from two individuals to a mixed DNA typing result. The use of quantitative peak height information can be used to calculate the mixture ratio (Butler, 2010a) from loci containing the maximum number of alleles (four alleles in mixtures from two unrelated individuals).

Table 27 - Mixture ratios obtained using the five available heterozygous loci. The average of these mixture ratios is also represented as well as the peak height ratio concerning Amelogenin locus.

		FEMALE (MALE CONSTANT)					**Mixture ratios**		MALE (FEMALE CONSTANT)				
		20:1	**15:1**	**10:1**	**5:1**	**2:1**	**1:1 [M:1]**	**1:1 [F:1]**	**2:1**	**5:1**	**10:1**	**15:1**	**20:1**
Mixture ratio	D21S11	-	7:1	-	6:1	2:1	1:1	2:1	2:1	11:1	14:1	11:1	16:1
Mixture ratio	D1S1656	-	-	-	4:1	1:1	2:1	1:1	1:1	7:1	16:1	-	-
Mixture ratio	D12S391	19:1	37:1	8:1	5:1	1:1	2:1	1:1	2:1	7:1	7:1	20:1	24:1
Mixture ratio	FGA	18:1	22:1	9:1	7:1	2:1	1:1	1:1	2:1	5:1	10:1	13:1	13:1
Mixture ratio	D18S51	-	5:1	10:1	5:1	1:1	1:1	1:1	3:1	9:1	10:1	15:1	13:1
Average		**19:1**	**18:1**	**9:1**	**5:1**	**1:1**	**1:1**	**1:1**	**2:1**	**8:1**	**11:1**	**15:1**	**17:1**
Peak Height Ratio	AMEL	-	2%	7%	13%	21%	23%	31%	33%	84%	97%	99%	74%

Mixture ratios can help deduce contributor profiles (Butler, 2010a, SWGDAM, 2010). In Table 27 it is possible to observe that when mixture ratios reached high levels of disproportionality (equal or higher than 1:10) it becomes very difficult to achieve a reliable proportion of contributors. The red values represent ratios with the highest deviation from the real mixture ratio. The peak height ratio of Amelogenin locus was estimated in order to observe the behaviour of this marker in a possible male/female mixture. As expected, when the male DNA was constant with an increase in female DNA, the imbalance in this locus was progressively higher (from 1:1 to 20:1). On the other hand, concerning the other experience where female DNA was constant with an increase in male DNA, the Amelogenin had an imbalance increasingly smaller (1:1 to 20:1).

4.6. Precision Study

Accurate and reliable genotypes can be obtained by sizing precision studies. The method that has been recommended for this kind of analysis (Applied Biosystems,

2011) is to employ a ±0.5-nt "window" around the size obtained for each allele in the Investigator® ESSplex Plus Allelic Ladder.

This study was made taking into account the fact that when GeneMapper® ID Software automatically flags an allele that does not size within the prescribed window around an allelic ladder, labelling it as OL, this has to be confirmed, if in doubt, by repeating the analysis and not only considering the precision sizing values obtained in this internal validation work.

This experiment evaluated the allele size variances obtained from multiple injections of the allelic ladder and assessed the use of GeneMapper® ID Software (v.3.2) on designating alleles. Data for each allele was analysed by determining their sizes in base pairs and the average and SD for each locus of the ESSplex Plus was calculated.

In this study two different perspectives were analysed: under controlled conditions and unintentionally varying conditions. The latter results were mainly from room temperature fluctuations during time, but also from other factors such as variations in ABI routine maintenance.

The charts for each locus (below) exhibit SD bin sizes for each allele within that locus (Sgueglia *et al.*, 2003).

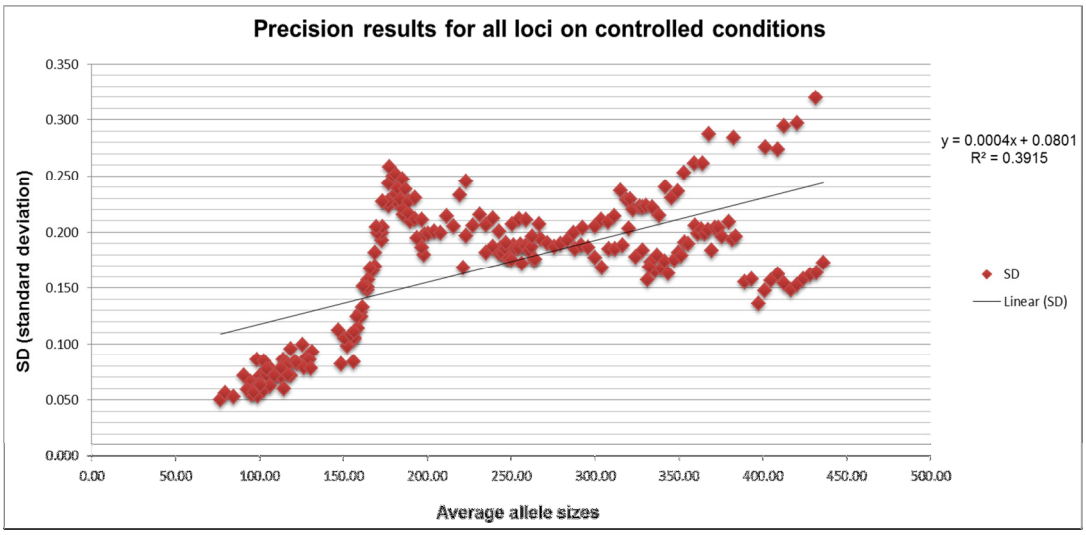


Figure 60 - Precision results for the study in controlled conditions, where all loci of the ESSplex Plus kit were analyzed together, with respect to their allele sizes and SD.

Maximum sizing precision is obtained within the same set of capillary injections with fewer changes in the involved conditions. In this way, concerning the study where the conditions were apparently controlled (Figure 60), there seems to exist a relation between the increasing of the dispersion with increasing size of the marker. It was also relevant the fact that the maximum SD was lower than the 0.5 bp reference value, which demonstrated very good precision with bin sizes approximately equal to or less than 0.32 bp (maximum SD was approximately 0.319 bp for the FGA locus). The FGA locus was responsible for the highest sizing differences as already mentioned in other studies (Applied Biosystems, 2011, Sgueglia *et al.*, 2003). The locus with the lowest average SD was Amelogenin with 0.050 base pair deviation.

However, sizing differences occur due to a number of factors including different polymer lots, run temperature, electrophoresis conditions, and room temperature. Therefore, it was also evaluated a set of ladder injections performed on different days in week spans, where different conditions are expected (Figure 61). The results show that there is also a relation between the increase of the dispersion and the increase in marker size. However, the maximum SD reflected in uncontrolled conditions occurred substantially above the 0.5 bp recommended window (maximum SD was approximately 1.215 bp in the FGA locus) (Figure 61). The locus with the lowest average SD was TH01 with 0.081 base pair deviation (Figure 61).

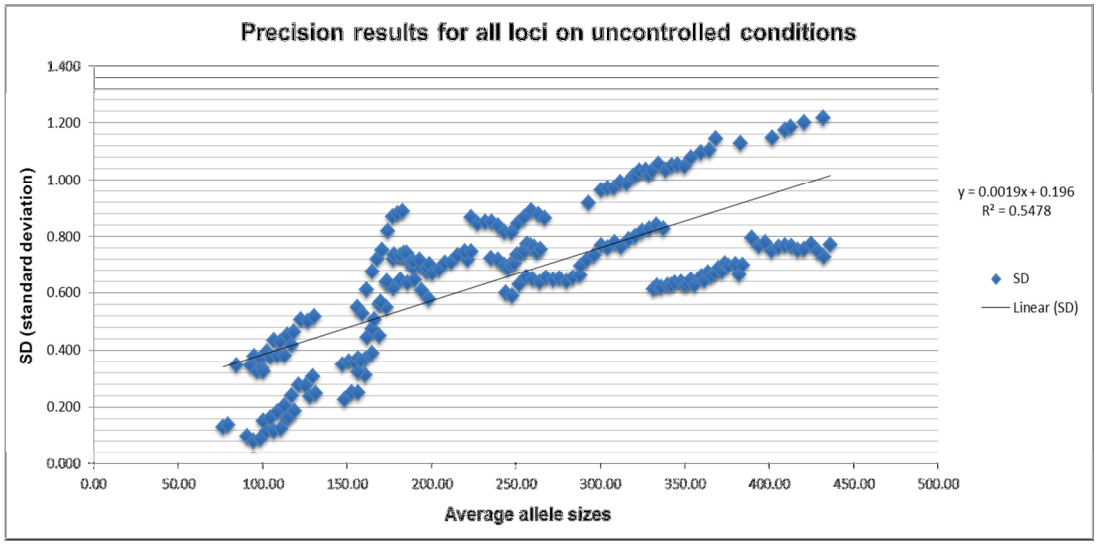


Figure 61 - Precision results for the study in uncontrolled conditions, where all loci of the ESSplex Plus kit were analyzed together, with respect to their allele sizes and SD.

In Figure 62, all ladder injections are graphically represented in both situations: controlled and uncontrolled studies.

Thus, taking into account all the results obtained for ESSplex Plus system, caution is needed especially when larger alleles of particular loci (such as FGA) are interpreted, in order to avoid mistakes concerning, for instance, mistaking variant alleles for on-ladder ones (Sgueglia *et al.*, 2003).

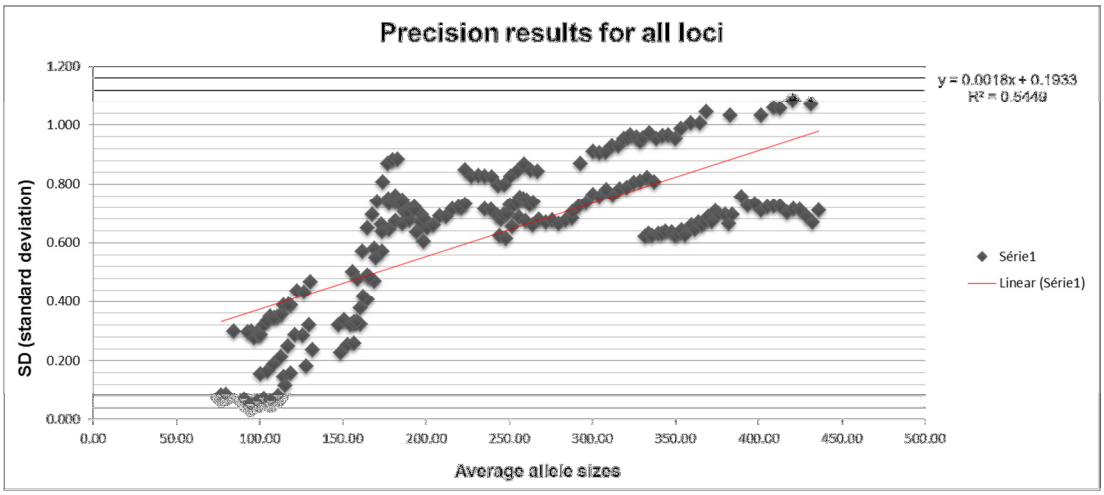


Figure 62 - Precision results for the study with uncontrolled and controlled conditions, where all loci of the ESSplex Plus kit were analyzed together, respecting their sizes and SD.

Individual analysis regarding the set of loci that compose the ESSplex Plus kit was also performed, in order to detect any peculiarity in what concerns precision behaviour vs size and/or structure.

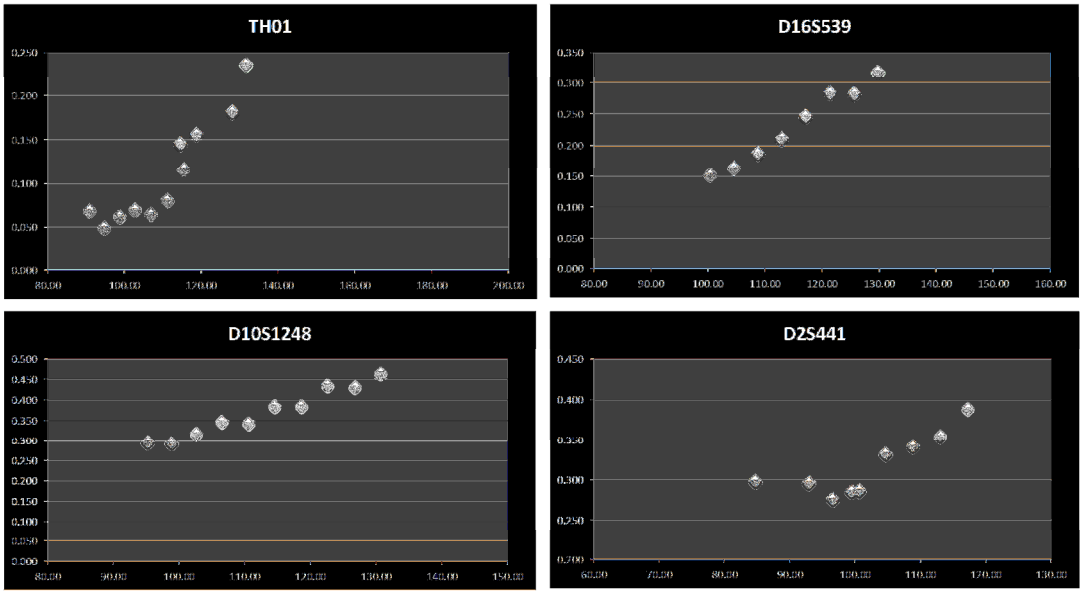


Figure 63 - Precision results for TH01, D16S539, D10S1248 and D2S441 markers. These distributions resulted from the study where values of the controlled and uncontrolled experiments were considered.

In Figure 63, the smaller sized loci from ESSplex Plus kit are represented. This set of markers showed a more homogeneous behaviour, less disperse and with a more proportional distribution than larger loci, which is most probably due to their fragment size.

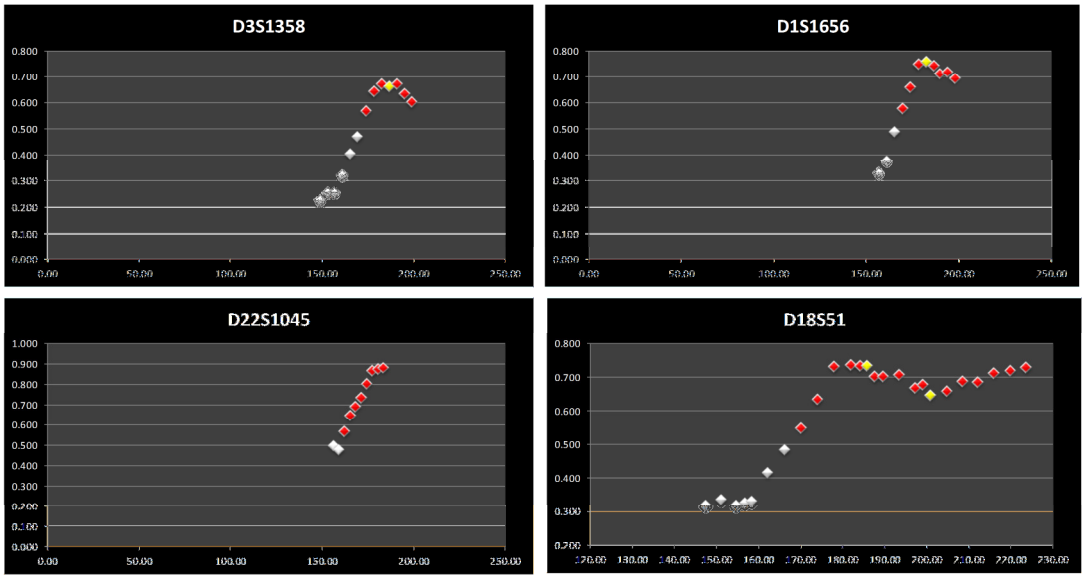


Figure 64 - Precision results for D3S1358, D1S1656, D22S1045 and D18S51markers. These distributions resulted from the study where values of the controlled and uncontrolled experiments were considered.

In Figure 64, the loci in the range of 130 – 240 bp are shown. Approximately after 170bp is the point where the precision values exceed the 0.5 bp recommended. Therefore, represented by the white colour are the values below the 0.5 bp window and the red colour represents the values that are above. There are also values symbolized by yellow points where the marker distribution changes. There is a dispersion decrease observed at D3S1358, D1S1656 and D18S51 around 180-190bp (no D22S1045 alleles analysed at this range), and it increases again after the 200bp point (shown by D18S51). There is no evident explanation for this behaviour for all the loci in this size range.

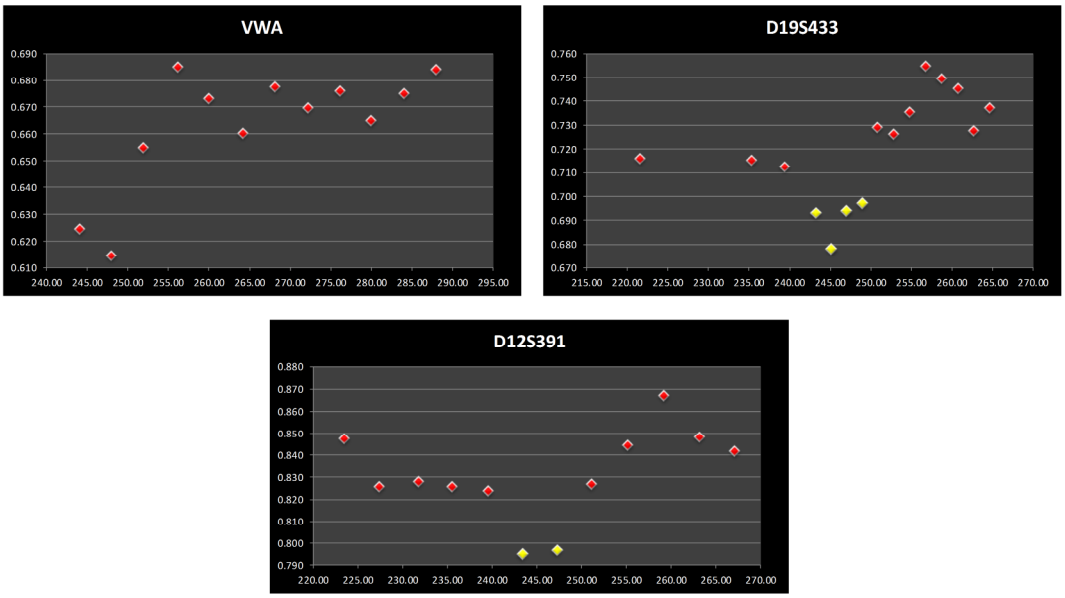


Figure 65 - Precision results for VWA, D19S433 and D12S391 markers. These distributions resulted from the study where values of the controlled and uncontrolled experiments were considered.

In Figure 65, a particular behaviour concerning the D19S433 and the D12S391 loci can be observed. More exactly at the 240 – 260 bp range, these two markers show a marked decrease in the dispersion event (yellow points). Since this decrease is followed by a new increase in the dispersion values, an isolated effect must be associated. In this case, a possible explanation for the smaller dispersion detected at this size range may relate to the size standard (550 BTO, Figure 66) which at this point has fragments with a 10 bp interval instead of the usual 20 bp interval present throughout this size standard.

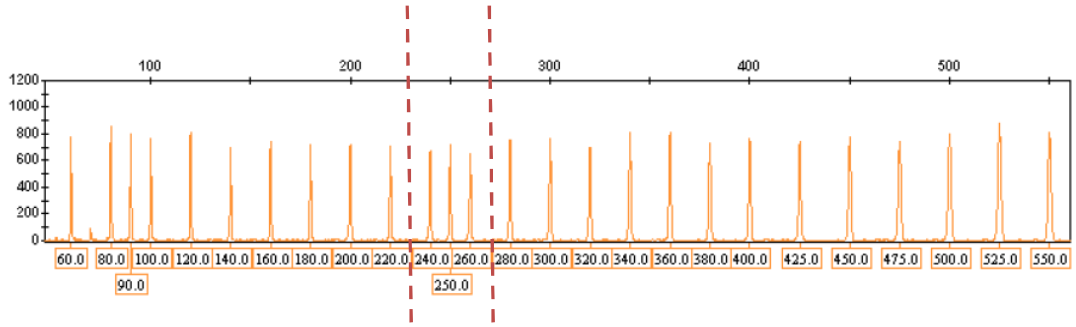


Figure 66 - Electropherogram of the DNA Size Standard 550 (BTO), fragments with lengths in bp (Qiagen, 2011b). The 240 – 260 bp range is delimited as an example of 10 bp intervals in the 550 BTO size standard.

In Figure 67, three loci in the same size range with similar behaviour in their distribution are shown. These markers reflect the dispersion behaviour at higher size ranges. Here, it is evident the direct proportional relationship between dispersion increase and increasing size of the marker.

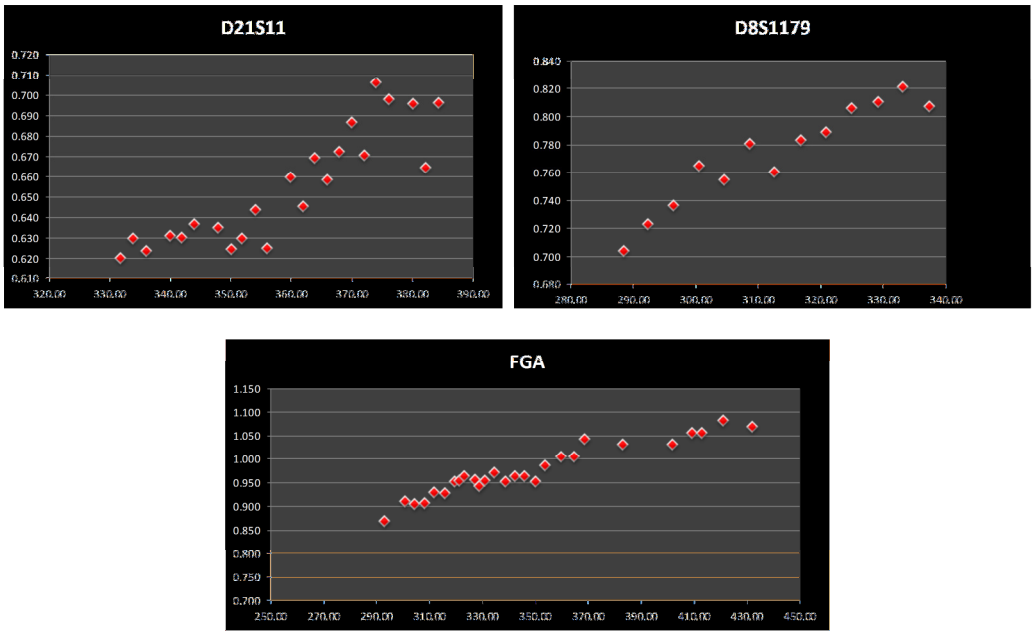


Figure 67 - Precision results for D21S11, D8S1179 and FGA markers. These distributions resulted from the study where values of the controlled and uncontrolled experiments were considered.

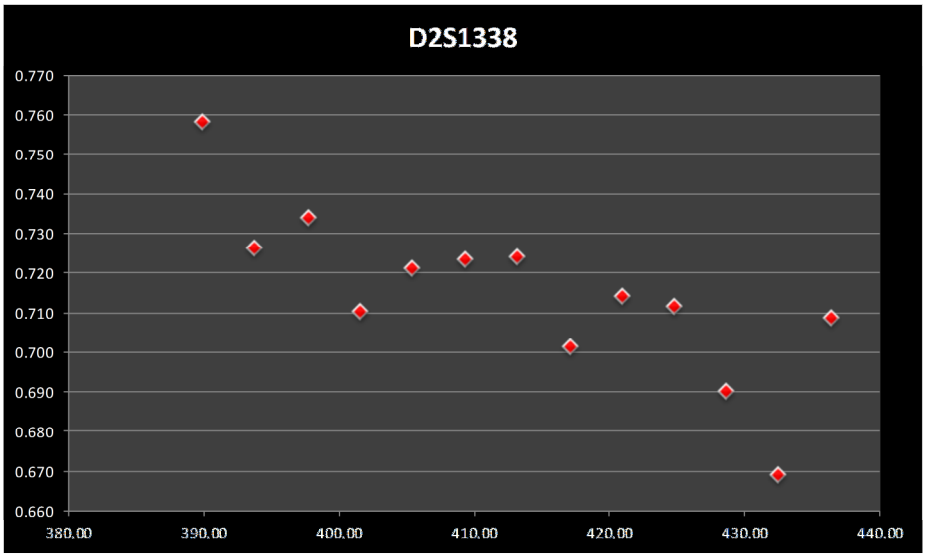


Figure 68 - Precision results for D2S1338 marker. This distribution resulted from the study where values of the controlled and uncontrolled experiments were considered.

In Figure 68, the dispersion concerning the D2S1338 marker is represented, which is markedly different from other markers of the ESSplex Plus system. The D2S1338 locus shows a completely different dispersion since, concerning its size (380 to 440 bp), it would be expectable to observe an increase of its dispersion values through the allelic range. It is difficult to find an explanation for this marked decrease in the dispersion values, since it is not known if any single factor is causing this effect. Although this is the only marker mainly inside the size standard region where sizing fragments have a 25bp interval, this would not explain lower dispersion; on the contrary, larger dispersion would be expected.

This new next generation kit used with the 550 BTO size standard has revealed the precision requirements necessary to conduct forensic DNA analysis using an allelic ladder to size the unknown fragments. In the same way as in all multiplex PCR kits, it requires the repeated use of the allelic ladder in several runs, since temperature fluctuations, along with other factors, interfere with electrophoresis runs. If severe temperature fluctuations exist, as well as changes in other conditions, it is advisable to run an allelic ladder in each run (Sgueglia *et al*., 2003).

4.7. Degradation Study

In order to evaluate the newly developed miniSTR systems, tests were conducted on artificially degraded DNA samples, for the four different multiplex kits: ESSplex Plus, NGM, Identifiler Plus and Powerplex HS 16. The first two systems present the 5 new loci which are frequently announced as new potential loci for analysis of degraded DNA.

Since AmpFlSTR® NGM™ kit has the same mini-STR loci as Investigator® ESSplex Plus kit, a comparative analysis was made in order to evaluate the performance and capability of detection of the maximum genetic information content, consisting in the evaluation of the number of heterozygotes detected.

Nevertheless, Identifiler® Plus Kit and PowerPlex® 16 HS Kit were also tested for this validation study since it seems crucial to evaluate the performance of the smaller genetic markers incorporated in these multiplexes which are currently in use in the laboratory, and if, in fact, the 5 new mini-STRs present in the recent kits do make a difference.

Therefore, the behaviour of the four systems were evaluated and compared in order to observe if some advantage exists in the use of ESSplex Plus or NGM systems. This approach gave us a better ability to choose the autosomal kit that may provide us more genetic information, in better conditions, for our routine work.

The method of analysis consisted in the evaluation and identification of the heterozygous loci that were amplified with DNA UV exposed from 0 to 20 minutes, in the following time intervals: 20'', 40'', 1', 3', 10', 15' and 20'. The maximum time of exposure that our sample underwent corresponds to the recommended time of exposure for DNA decontamination in laminar flow or PCR cabinets.

The analysis of data was also done using both approaches, previously established: visual and threshold approaches. The heterozygous loci number, across the degradation time range, were converted to a percentage value to facilitate comparison between the four systems analysed (these are composed by a different number and type of loci). With the visual approach, these heterozygous loci were selected, independently of any threshold. On the other hand, with the threshold approach, only the heterozygous loci that were selected by the software of analysis were considered.

Concerning the comparison between the two different approaches, the threshold approach demonstrated more conservative results since it revealed lower percentages of heterozygous loci detected. Also, with this sort of approach, there is greater heterogeneity of values among the different multiplexes, for each UV exposure time (Figure 70).

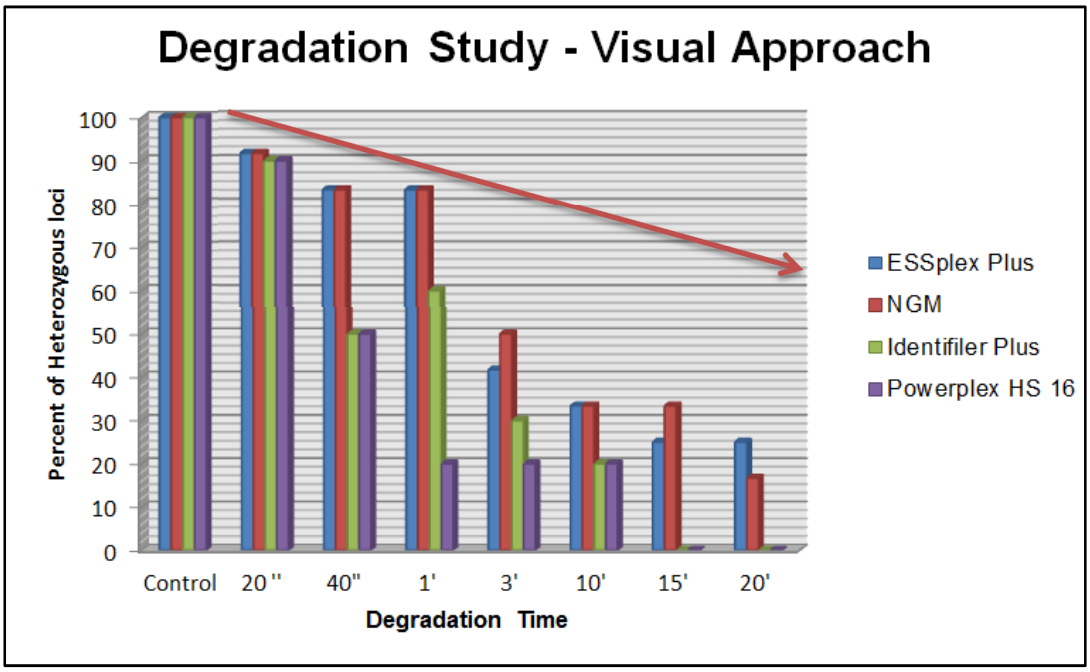


Figure 69 - Degradation study results concerning the visual approach. The percentage of heterozygous loci detected across the time range of degradation exposure was calculated for the four multiplexes in study.

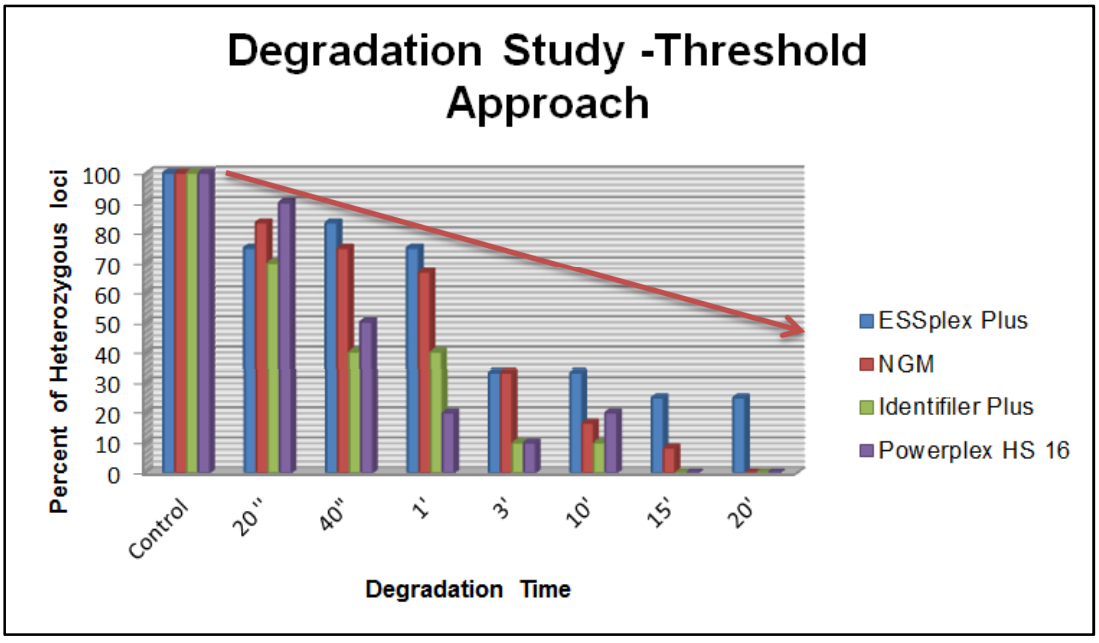


Figure 70 - Degradation study results concerning the threshold approach. The percentage of heterozygous loci detected across the time range of degradation exposure was calculated for the four multiplexes in study.

Concerning the comparison between multiplex system performance and regarding the visual approach, the greatest loss of heterozygous loci happened at different degradation times. For the Identifiler Plus and Powerplex HS 16 kits, at 40 seconds of UV exposure there was a loss of 50% of the initial amount of heterozygous loci (Figure 69). On the other hand, for ESSplex Plus and NGM kits, the same loss only happened at 3 minutes of exposure (Figure 69).

Therefore, the ESSplex Plus and NGM systems presented, through the visual approach, a higher degree of informativeness in comparison with Identifiler Plus and Powerplex HS 16, and a similar degree between them regarding the number of heterozygous loci detected. These two kits are also the only that ensure some kind of genetic information above the 15 minutes of UV exposure (Figure 69).

Still, concerning the Identifiler Plus and Powerplex kits, not only did they show a more rapid loss of heterozygous loci but also did not allow for any genetic information at 15 and 20 minutes of UV exposure (Figure 69).

With the threshold approach, the degradation effect among these four systems followed more or less the same behaviour. However, the NGM kit was unable to ensure any genetic information at 20 minutes of UV exposure (Figure 70), unlike the visual approach, and it can be clearly shown a better performance for the ESSplex Plus system. Therefore, the ESSplex Plus kit, in this study, is the only system capable of delivering heterozygous detection at the highest level of degradation (20 minutes), and is the system that gives generally the highest genetic information throughout the degradation process Figure 71.

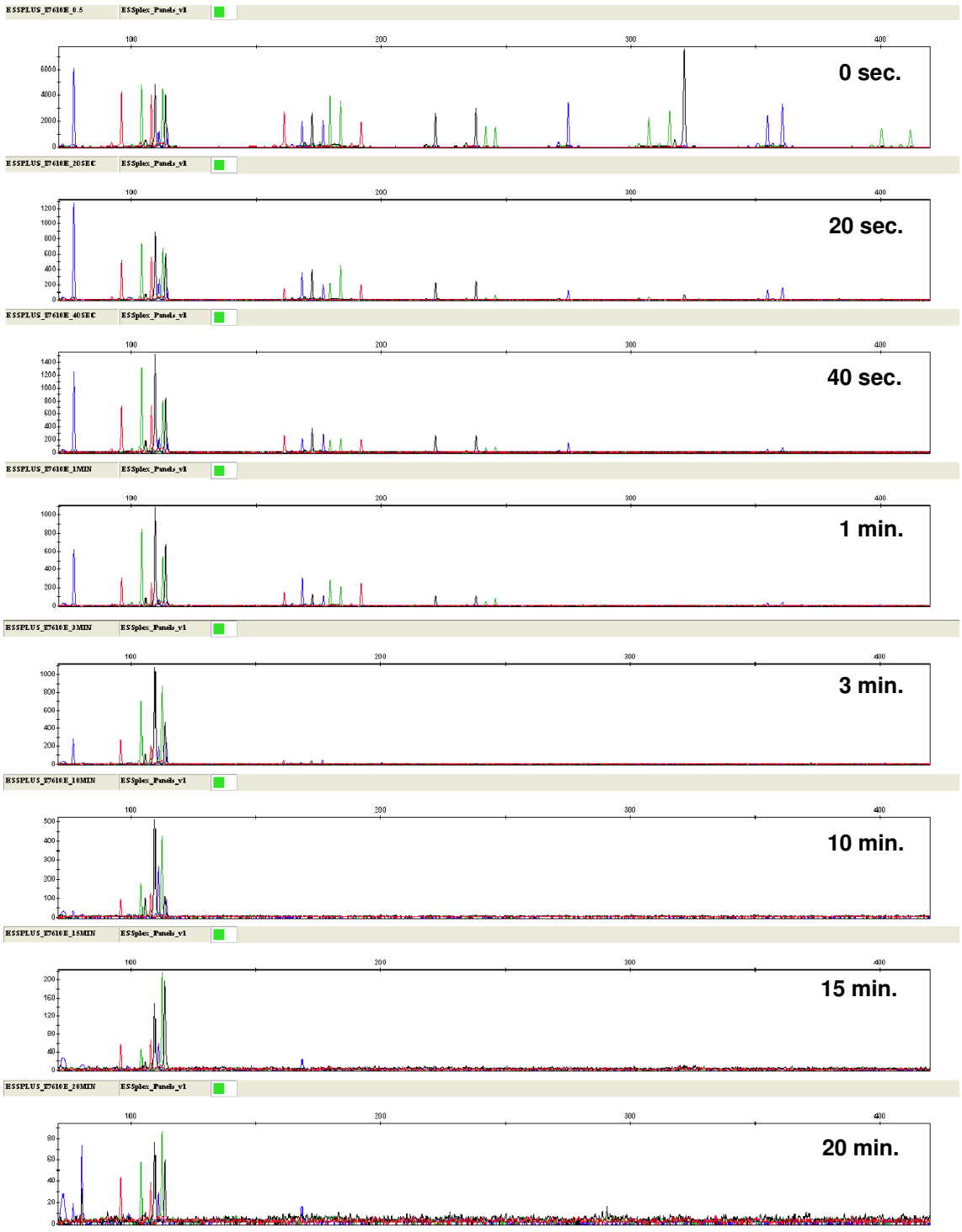


Figure 71 - Example of an electropherogram of a random sample submitted to different times of UV-light exposure analysed with ESSplex Plus kit. In this picture, the values of time exposure, experimented in the degradation test (from 0 seconds to 20 minutes), are represented. Note that the y- axis scale was magnified for the smaller input amounts of DNA.

5. CONCLUSIONS

Nowadays, Human STR genotyping for forensic analyses and population studies is currently done with commercially available autosomal STR multiplex kits, the majority of which amplify a minimum of 15 markers in a single PCR. However, a new ESS of forensic genetic makers was proposed to be adopted across the European scientific community. In this way, the internal validation study of a new next generation kit, having this new set, is critical in order for it to be applied in the laboratory's forensic casework. The kits currently used, Identifiler Plus and Powerplex 16 HS, lack these new five ESS STRs and so a new multiplex kit must be implemented and substitute one of the latter. The ESSplex Plus kit was primarily chosen for this goal, since Qiagen has demonstrated consistency and robustness in their products throughout the years and have shown very competitive prices in their recent launch of human identity products.

The validation of the five new loci in the Portuguese population revealed no deviations from Hardy-Weinberg equilibrium. The robustness of forensic parameters concerning this new set and the increased discrimination power provided by this new ESS will also assist in paternity testing and complex relationship elucidation. It will allow improving the power of the "in-house" database whilst evolving to the current European standards.

The comparison of our Portuguese sample with other European samples, available in the literature, generally revealed no significant allele frequency differences for the five new loci. This implies, on one hand, that genotyping was well carried out and, on the other hand, that a general European database could be used and information exchange throughout Europe can be correctly accomplished. It is important to note that ensuring the same nomenclature and robust laboratory techniques across European population studies is important and over the years this has been aided and achieved through robust commercial multiplex kits.

In fact, despite using different primer sequences, 100% concordance between the same 15 autosomal loci in ESSplex Plus and the NGM system was demonstrated.

Through the contamination study, it was possible to estimate a common Analytical Threshold of 30 RFU for ESSplex Plus, NGM and the two previously used multiplex systems, showing a marked improvement at the baseline level.

Two different approaches were used for analysing genotype data. The visual approach, where the expert's experience is the only opinion that counts, showed different results when compared with the threshold approach, where analytical and stochastic thresholds are applied as well as estimated parameters of stutter and peak height ratios. This was expected in the same way as it was expected that this last approach would reveal to be more conservative. This demonstrates and confirms that if the visual approach were to be carried out by more than one expert, the results would most certainly be different and difficulty in defining a final interpretation would occur. It confirms the advantages of using thresholds to facilitate interpretation bias, although it should be emphasized that this will most probably lead to loss of genetic information. In fact, when dealing with mixtures with clear minor contributor(s), the threshold approach will undoubtedly eliminate that information.

The ESSplex Plus was designed for maximal sensitivity under the cycling conditions recommended for these multiplexes and for robust amplification in the presence of high levels of common PCR inhibitors. Under recommended cycling and injection conditions ESSplex Plus kit showed more sensitivity compared to NGM kit and the routinely used Identifiler Plus and Powerplex 16 HS systems. Thus, optimal profile balance was achieved at 0.5 ng of input DNA, which is in accordance with Qiagen's recommended values. The ESSplex Plus kit also demonstrated a higher capability in genetic information detection, at lower levels of DNA amount.

The individual estimation of stutter ratio values for each ESSplex Plus locus will contribute to improve mixture analysis. The mixture of two contributors in this study demonstrated that the capability of this system in detecting the minor contributing alleles is at 1:20 ratios. The evaluation of the power of this system in estimating the ratio of DNA contributors to a mixed DNA typing result when two contributors are involved, revealed that in mixture ratios equal or higher than 1:10 it is difficult to achieve a reliable proportion of contributors.

The precision results concerning ESSplex Plus system has shown that electrophoretic mobility is totally dependent on environment fluctuations over time, and allele sizing and designation may be compromised. The allele precision behaviour for each STR that was studied revealed that, apart from some isolated cases, in general, smaller sized loci have more precise sizing than larger loci; that smaller alleles in a locus will have more precision than larger ones; and that there is also a strong influence in the

size standard in what concerns its fragments size intervals (i.e. smaller intervals will lead to more precision).

Degraded samples amplified with ESSplex Plus system in this study highlighted than an improved recovery of genetic information can be achieved compared with the remaining multiplexes (NGM, Identifiler Plus and Powerplex 16 HS). The success of the ESSplex Plus system in this study can be attributed, in part, to improved sensitivity (Tucker *et al*., 2011a) plus the increased number of mini-STRs.

The internal validation studies presented here illustrate the improved performance of the ESSplex Plus multiplex compared with the standard Identifiler Plus and Powerplex 16 HS, routinely used.

Future analysis using ESSplex Plus kit as one of the multiplex systems incorporated in forensic casework routine should provide, by the use of real time PCR quantification analysis, a better management of the input DNA used as well as the laboratory reagents involved.

Since the internal validation study has been completed, IPATIMUP's laboratory of genetic identification and kinship analysis has now the ability to develop internal interpretation guidelines based on the results of all the studies carried out. Therefore, along with the developmental validation study performed by Qiagen, these internal validation studies provide concrete information about ESSplex Plus performance, which can be a useful tool in future DNA analysis (Tucker *et al*., 2011b).

Results of our validation study demonstrates that the ESSplex Plus kit is a reliable multiplex, which may be suitable for use on all types of forensic samples, since it has shown throughout this work to have robust PCR chemistry and improved performance, requested by the European forensic community for typing degraded samples and ensuring reliable results.

6. REFERENCES

ALBINSSON, L., NORÉN, L., HEDELL, R. & ANSELL, R. 2011. Swedish population data and concordance for the kits PowerPlex® ESX 16 System, PowerPlex® ESI 16 System, AmpF *l* STR® NGM™, AmpF *l* STR® SGM Plus™ and Investigator ESSplex. *Forensic Sci Int Genet,* 5, e89-e92.

ALVES, C. 2011. Technical procedure manual: DNA extraction by Chelex (LPIG). *In:* IPATIMUP (ed.).

ALVES, C., AMORIM, A. & GUSMÃO, L. 2001. VWA STR genotyping: further inconsistencies between Perkin-Elmer and Promega kits. *Int J Leg Med,* 115**,** 97-99.

ALVES, C., AMORIM, A., PEREIRA, L. & GUSMÃO, L. 2004. African STR data based on a newly developed tetraplex fluorescent system (CD4, F13A01, FES and MBPB). *Progress in Forensic Genetics 10,* 1261**,** 133-135.

ALVES, C., GUSMAO, L., PEREIRA, L. & AMORIM, A. 2003. Multiplex STR genotyping: comparison study, population data and new sequence information. *Progress in Forensic Genetics 9. Proceedings from the 19th International ISFG* 1239**,** 131-135.

AMORIM, A., ALVES, C., GUSMÃO, L. & PEREIRA, L. 2006. Extended Northern Portuguese database on 21 autosomal STRs used in genetic identification. *Progress in Forensic Genetics 11 — Proceedings of the 21st International ISFG* 1288**,** 364-366.

AMORIM, A., ALVES, C., PEREIRA, L. & GUSMÃO, L. 2004. Genotyping inconsistencies and null alleles using AmpFLSTR® Identifiler® and Powerplex® 16 kits. *Progress in Forensic Genetics 10,* 1261**,** 176-178.

APPLIED BIOSYSTEMS 2005. GeneMapper® ID Software for Human Identification Applications, v.3.2.

APPLIED BIOSYSTEMS 2010. 7500 Fast Real-Time PCR System, v.2.0.5.

APPLIED BIOSYSTEMS 2011. AmpFlSTR® NGM™ PCR Amplification Kit - USER GUIDE.

APPLIED BIOSYSTEMS 2012. AmpFlSTR® Identifiler® Plus PCR Amplification Kit - User´s Guide.

BAKKER, S., SINKE, R. & PEARSON, P. 2005. Differences in stutter intensities between microsatellites are related to length and sequence of the repeat. *In:* UTRECHT, U. M. C. (ed.). Section III- new genotyping techniques.

BÄR W., BRINKMANN B., BUDOWLE B., CARRACEDO A., GILL P., LINCOLN P., MAYR W. & B., O. 1997. DNA recommendations - further report of the DNA

Commission of the ISFG regarding the use of short tandem repeat systems. *Forensic Sci Int,* 87**,** 179-184.

BENDER, K., FARFÁN, M. J. & SCHNEIDER, P. M. 2004. Preparation of degraded human DNA under controlled conditions. *Forensic Sci Int,* 139**,** 135-140.

BERTI, A., BRISIGHELLI, F., BOSETTI, A., PILLI, E., TRAPANI, C., TULLIO, V., FRANCHI, C., LAGO, G. & CAPELLI, C. 2010. Allele frequencies of the new European Standard Set (ESS) loci in the Italian population. *Forensic Sci Int Genet,* 5**,** 548-549.

BOTSTEIN, D., WHITE, R. L., SKOLNICK, M. & DAVIS, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet,* 32**,** 314.

BREGU, J. 2011. *Investigation of Baseline Noise: Establishing an RFU Threshold for Forensic DNA Analysis.* Boston University.

BROOKES, C., BRIGHT, J. A., HARBISON, S. A. & BUCKLETON, J. 2011. Characterising stutter in forensic STR multiplexes. *Forensic Sci Int Genet,* 6**,** 58-63.

BUDOWLE, B., MORETTI, T. R., KEYS, K. M., KOONS, B. W. & SMERICK, J. 1997. Validation studies of the CTT STR multiplex system. *J Forensic Sci,* 42**,** 701-707.

BUDOWLE, B., ONORATO, A. J., CALLAGHAN, T. F., MANNA, A. D., GROSS, A. M., GUERRIERI, R. A., LUTTMAN, J. C. & MCCLURE, D. L. 2009. Mixture Interpretation: Defining the Relevant Features for Guidelines for the Assessment of Mixed DNA Profiles in Forensic Casework*. *J Forensic Sci,* 54**,** 810-821.

BUEL, E., SCHWARTZ, M. B. & LAFOUNTAIN, M. J. 1998. Capillary electrophoresis STR analysis: comparison to gel-based systems. *J Forensic Sci,* 43**,** 164-170.

BUTLER, J. M. 2005. *Forensic DNA typing: biology, technology, and genetics of STR markers*, Elsevier Academic Press.

BUTLER, J. M. 2006a. Debunking some urban legends surrounding validation within the forensic DNA community. 9.

BUTLER, J. M. 2006b. *RE: MiniSTRs:Past, Present and Future.*

BUTLER, J. M. 2006c. Validation Workshop. HID University/Future Trends in Forensic DNA Technology. *In:* NIST (ed.). Albany, NY.

BUTLER, J. M. 2007. *RE: Validation: What Is It, Why Does It Matter, and How Should It Be Done?*

BUTLER, J. M. ISHI 2010a. Mixture Interpretation Workshop: Principles, Protocols and Practice. Mixture Ratios, 2010 Boston University. Promega-NIST.

BUTLER, J. M. 2010b. NIST Update. *In:* EDNAP_ENFSI_NIST (ed.) *EDNAP and 32th ENFSI DNA WG Meeting.* Hague,Netherlands: NIST.

BUTLER, J. M. 2011a. DNA Mixture Analysis: Principles and Practice of Mixture Interpretation and Statistical Analysis Using the SWGDAM STR Interpretation Guidelines. *In:* NIST (ed.) *SWGDAM Guidelines and Mixture Literature.* Chicago, IL: NIST.

BUTLER, J. M. Foundations for Mixture Interpretation: ISFG and SWGDAM. Maryland Mixture Workshop, 2011b Baltimore. NIST:National Institute of Standards and Technology.

BUTLER, J. M. DNA Mixture Interpretation & Statistical Analysis. Training Seminar, 2012 CIB Forensic Science Center:Taipei, Taiwan. NIST: National Institute of Standards and Technology.

BUTLER, J. M. & HILL, C. R. 2012. Biology and Genetics of New Autosomal STR Loci Useful for Forensic DNA Analysis. *Forensic Sci Rev,* 24**,** 16-26.

BUTLER, J. M., TOMSEY, C. S. & KLINE, M. C. Can the validation process in forensic DNA typing be standardized. 15th International Symposium on Human Identification, 2004. 1-20.

CLARK, J. M. 1988. Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acids Res,* 16**,** 9677-9686.

CLAYTON, T., WHITAKER, J., SPARKES, R. & GILL, P. 1998. Analysis and interpretation of mixed forensic stains using DNA STR profiling. *Forensic Sci Int,* 91**,** 55-70.

COBLE, M. D. Topics and Techniques for Forensic DNA Analysis: Mixture Interpretation. Continuing Education Seminar, 2012 New York City. NIST:National Institute of Standards and Technology.

COBLE, M. D. & BUTLER, J. M. 2005. Characterization of new miniSTR loci to aid analysis of degraded DNA. *J Forensic Sci,* 50**,** 43-53.

COLLINS, P. J., HENNESSY, L. K., LEIBELT, C. S., ROBY, R. K., REEDER, D. J. & FOXALL, P. A. 2004. Developmental validation of a single-tube Amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFSTR® Identifiler® PCR Amplification Kit. *J Forensic Sci,* 49**,** 1265-1277.

CORTELLINI, V., CERRI, N. & VERZELETTI, A. 2011. Population data on 5 non-CODIS STR loci (D10S1248, D22S1045, D2S441, D1S1656, D12S391) in a population sample from Brescia county (Northern Italy). *Forensic Sci Int Genet,* 5**,** e97-e98.

COTTON, E., ALLSOP, R., GUEST, J., FRAZIER, R., KOUMI, P., CALLOW, I., SEAGER, A. & SPARKES, R. 2000. Validation of the AMPF*l*STR® SGM Plus™ system for use in forensic casework. *Forensic Sci Int,* 112**,** 151-161.

DIEGOLI, T. M., FARR, M., CROMARTIE, C., COBLE, M. D. & BILLE, T. W. 2011. An optimized protocol for forensic application of the PreCR™ Repair Mix to multiplex STR amplification of UV-damaged DNA. *Forensic Sci Int Genet,* 6**,** 498-503.

DIXON, L., DOBBINS, A., PULKER, H., BUTLER, J., VALLONE, P., COBLE, M., PARSON, W., BERGER, B., GRUBWIESER, P. & MOGENSEN, H. S. 2006. Analysis of artificially degraded DNA using STRs and SNPs—results of a collaborative European (EDNAP) exercise. *Forensic Sci Int,* 164**,** 33-44.

DOGNAUX, S., LARMUSEAU, M. H. D., JANSEN, L., HEYLEN, T., VANDERHEYDEN, N., BEKAERT, B., NOEL, F. & DECORTE, R. 2011. Allele frequencies for the new European Standard Set (ESS) loci and D1S1677 in the Belgian population. *Forensic Sci Int Genet,* 6**,** e75-e77.

EXCOFFIER, L. & LISCHER, H. E. L. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour,* 10**,** 564-567.

FBI 2008. Quality assurance standards for forensic DNA testing laboratories. *In:* COMMUNICATIONS, F. S. (ed.).

FISHER, R. A. 1951. Standard calculations for evaluating a blood-group system. *Heredity,* 5**,** 95-102.

FORMOSO, L., PHILLIPS, C., RODRIGUEZ, A. & CALVO, R. 2012. Allele frequencies of 20 STRs from Northwest Spain (Galicia). *Forensic Sci Int Genet,* 6**,** e149-e150.

GEHRIG, C., VUICHARD, S., TEYSSIER, A. & CASTELLA, V. 2011. Validation of the NGM SElect™ kit. *Progress in Forensic Genetics 14 — Proceedings of the 24th International ISFG,* 3**,** e453-e454.

GILDER, J. R., INMAN, K., SHIELDS, W. & KRANE, D. E. 2011. Magnitude-dependent variation in peak height balance at heterozygous STR loci. *Int J Leg Med,* 125**,** 87-94.

GILL, P., FEREDAY, L., MORLING, N. & SCHNEIDER, P. M. 2006a. The evolution of DNA databases—recommendations for new European STR loci. *Forensic Sci Int,* 156**,** 242-244.

GILL, P., FEREDAY, L., MORLING, N. & SCHNEIDER, P. M. 2006b. New multiplexes for Europe—amendments and clarification of strategic development. *Forensic Sci Int,* 163**,** 155-157.

GILL, P., PUCH-SOLIS, R. & CURRAN, J. 2009. The low-template-DNA (stochastic) threshold--Its determination relative to risk analysis for national DNA databases. *Forensic Sci Int Genet,* 3**,** 104-111.

GOODWIN, W., LINACRE, A. & HADI, S. 2011. *An introduction to forensic genetics*, Wiley.

GUO, S. W. & THOMPSON, E. A. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics,* 48**,** 361-372.

GUSMÃO, L. & ALVES, C. 2005. Y chromosome STR typing. *Methods Mol Biol,* 297**,** 67-82.

GUSMÃO, L., ALVES, C., GOMES, I. & SÁNCHEZ-DIZ, P. 2012. Capillary Electrophoresis of an X-Chromosome STR Decaplex for Kinship Deficiency Cases. *Methods Mol Biol,* 830**,** 57-71.

HATZER-GRUBWIESER, P., BERGER, B., NIEDERWIESER, D. & STEINLECHNER, M. 2011. Allele frequencies and concordance study of 16 STR loci-including the new European Standard Set (ESS) loci-in an Austrian population sample. *Forensic Sci Int Genet,* 6**,** e50-e51.

HILL, C. R., KLINE, M. C., MULERO, J. J., LAGACE, R. E., CHANG, C. W., HENNESSY, L. K. & BUTLER, J. M. 2007. Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J Forensic Sci,* 52**,** 870-873.

KIMPTON, C., FISHER, D., WATSON, S., ADAMS, M., URQUHART, A., LYGO, J. & GILL, P. 1994. Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci. *Int J Leg Med,* 106**,** 302-311.

KLINE, M. C., HILL, C. R., DECKER, A. E. & BUTLER, J. M. 2011. STR sequence analysis for characterizing normal, variant, and null alleles. *Forensic Sci Int Genet,* 5**,** 329-332.

KRENKE, B. E., TEREBA, A., ANDERSON, S. J., BUEL, E., CULHANE, S., FINIS, C. J., TOMSEY, C. S., ZACHETTI, J. M., MASIBAY, A. & RABBACH, D. R. 2002. Validation of a 16-locus fluorescent multiplex system. *J Forensic Sci,* 47**,** 773-785.

LAGOA, A. M., MARTINS, T. V., CAINÉ, L. M. & PINHEIRO, M. F. 2008. Allele frequencies of six miniSTR loci in the population of Northern Portugal. *Forensic Sci Int Genet,* 2**,** 379-381.

LAREU, M., BARRAL, S., SALAS, A., PESTONI, C. & CARRACEDO, A. 1998. Sequence variation of a hypervariable short tandem repeat at the D1S1656 locus. *Int J Leg Med,* 111**,** 244-247.

LAREU, M., PESTONI, C., CARRACEDO, A., SCHÜRENKAMP, M., RAND, S. & BRINKMANN, B. 1996. A highly variable STR at the D12S391 locus. *Int J Leg Med,* 109**,** 134-138.

LAREU, M., PHILLIPS, C., CARRACEDO, A., LINCOLN, P. & THOMSON, J. 1994. Investigation of the STR locus HUMTH01 using PCR and two electrophoresis formats: UK and Galician Caucasian population surveys and usefulness in paternity investigations. *Forensic Sci Int,* 66**,** 41-52.

LINS, A. M., SPRECHER, C. J., PUERS, C. & SCHUMM, J. W. 1996. Multiplex Sets for the Amplification of Polymorphic Short Tandem Repeat Loci--Silver Stain and Fluorescence Detection. *BioTechniques,* 20**,** 882-889.

MARTÍN, P., GARCÍA, O., ALBARRÁN, C., GARCÍA, P., YURREBASO, I. & ALONSO, A. 2007. Allele frequencies of six miniSTR loci (D10S1248, D14S1434, D22S1045, D4S2364, D2S441 and D1S1677) in a Spanish population. *Forensic Sci Int,* 169**,** 252-254.

MOLNÁR, A., ZALÁN, A., HORVÁTH, G. & PAMJAV, H. 2011. Allele distribution of the new European Standard Set (ESS) loci in the Hungarian population. *Forensic Sci Int,* 5**,** 555-556.

MORETTI, T. R., BAUMSTARK, A. L., DEFENBAUGH, D. A., KEYS, K. M., SMERICK, J. B. & BUDOWLE, B. 2001. Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. *J Forensic Sci,* 46**,** 647-660.

MORLING, N., ALLEN, R. W., CARRACEDO, A., GEADA, H., GUIDET, F., HALLENBERG, C., MARTIN, W., MAYR, W. R., OLAISEN, B. & PASCALI, V. L. 2002. Paternity Testing Commission of the International Society of Forensic Genetics: recommendations on genetic investigations in paternity cases. *Forensic Sci Int,* 129**,** 148-157.

MULERO, J. J., CHANG, C. W., LAGACE, R. E., WANG, D. Y., BAS, J. L., MCMAHON, T. P. & HENNESSY, L. K. 2008. Development and Validation of the AmpFℓSTR® MiniFilerTM PCR Amplification Kit: A MiniSTR Multiplex for the Analysis of Degraded and/or PCR Inhibited DNA*. *J Forensic Sci,* 53**,** 838-852.

NEI, M. 1987. *Molecular evolutionary genetics*, Columbia Univ Pr, New York.

PEREIRA, R., PHILLIPS, C., ALVES, C., AMORIM, A., CARRACEDO, Á. & GUSMÃO, L. 2009. A new multiplex for human identification using insertion/deletion polymorphisms. *Electrophoresis,* 30**,** 3682-3690.

PREVIDERÈ, C., GRIGNANI, P. & PRESCIUTTINI, S. 2011. Italian population data for the new ENFSI/EDNAP loci D1S1656, D2S441, D10S1248, D12S391, D22S1045. The GeFI collaborative exercise and concordance study. *Progress in Forensic Genetics 14 — Proceedings of the 24th International ISFG* 3**,** e238–e239.

PRIETO, L., ZIMMERMANN, B., GOIOS, A., RODRIGUEZ-MONGE, A., PANETO, G., ALVES, C., ALONSO, A., FRIDMAN, C., CARDOSO, S., LIMA, G., ANJOS, M., WHITTLE, M., MONTESINO, M., CICARELLI, R., ROCHA, A., ALBARRÁN, C., DE PANCORBO, M., PINHEIRO, M., CARVALHO, M., SUMITA, D. & PARSON, W. 2011. The GHEP–EMPOP collaboration on mtDNA population data—A new resource for forensic casework. *Forensic Sci Int Genet,* 5(2)**,** 146-151.

PROMEGA 2009 - 2012. Promega Technical Manual: Powerplex® 16 HS System. Promega Corporation.

PUCH-SOLIS, R., KIRKHAM, A., GILL, P., READ, J., WATSON, S. & DREW, D. 2011. Practical determination of the low template DNA threshold. *Forensic Sci Int Genet,* 5**,** 422-427.

QIAGEN 2010. Qiagen® InvestigatorTM Validation Guide. Qiagen - Sample & Assay Technologies.

QIAGEN 2011a. Investigator® Quantiplex Handbook: for quantification of human DNA in forensic samples. Qiagen Sample & Assay Technologies.

QIAGEN 2011b. Qiagen® InvestigatorTM ESSplex Plus Handbook. Qiagen - Sample & Assay Technologies.

QIAGEN 2012. Developmental validation of the Investigator® ESSplex Plus Kit *Investigator ESSplex Plus Validation Report.* Qiagen - Sample & Assay Technologies.

REICHERT, M. & PAWLOWSKI, R. 2009. Population genetics of six miniSTR loci (D1S1677, D2S441, D4S2364, D10S1248, D14S1434, D22S1045) in a Polish population. *Leg Med,* 11**,** 147-148.

ROUSSET, F. 2010. GENEPOP 4.1.0 for Windows/Linux/Mac OS X. 4.1.0 ed.

SCHNEIDER, P. M. 2007. Scientific standards for studies in forensic genetics. *Forensic Sci Int,* 165**,** 238-243.

SCHNEIDER, P. M. 2009. Expansion of the European Standard Set of DNA Database Loci—the Current Situation. *Profiles in DNA,* 12**,** 6-7.

SEIDER, T., FIMMERS, R., BETZ, P. & LEDERER, T. 2010. Allele frequencies of the five miniSTR loci D1S1656, D2S441, D10S1248, D12S391 and D22S1045 in a German population sample. *Forensic Sci Int Genet,* 4**,** e159-e160.

SGUEGLIA, J. B., GEIGER, S. & DAVIS, J. 2003. Precision studies using the ABI prism 3100 genetic analyzer for forensic DNA analysis. *Anal Bioanal Chem,* 376**,** 1247-1254.

SIVAK, S. 2011. Evaluating Stochastic Thresholds for Six Amplification Kits. *In:* THE BODE TECHNOLOGY GROUP, I. (ed.).

SPRECHER, C. J., PUERS, C., LINS, A. M. & SCHUMM, J. W. 1996. General approach to analysis of polymorphic short tandem repeat loci. *BioTechniques,* 20**,** 266-277.

SWGDAM. 2004. *Revised Validation Guidelines* [Online]. Forensic Sci. Comm. Available: http://fbi.gov/hq/lab/fsc/backissu/july2004/standards/2004_03_ standards 02.htm.

SWGDAM 2010. SWGDAM Interpretation Guidelines for Autosomal STR Typing by Forensic DNA Testing Laboratories (approved January 2010).

TAMARIZ, J., VOYNAROVSKA, K., PRINZ, M. & CARAGINE, T. 2006. The application of ultraviolet irradiation to exogenous sources of DNA in plasticware and water for the amplification of low copy number DNA. *J Forensic Sci,* 51**,** 790-794.

THACKER, C., OGUZTURUN, C. & BALL, K. An investigation into methods to produce artificially degraded DNA. 2006. Elsevier, 592-594.

THANGARAJ, K., REDDY, A. & SINGH, L. 2002. Is the amelogenin gene reliable for gender identification in forensic casework and prenatal diagnosis? *Int J Leg Med,* 116**,** 121-123.

TUCKER, V. C., HOPWOOD, A. J., SPRECHER, C. J., MCLAREN, R. S., RABBACH, D. R., ENSENBERGER, M. G., THOMPSON, J. M. & STORTS, D. R. 2011a. Developmental validation of the PowerPlex® ESI 16 and PowerPlex® ESI 17 Systems: STR multiplexes for the new European standard. *Forensic Sci Int Genet,* 5**,** 436-448.

TUCKER, V. C., KIRKHAM, A. J. & HOPWOOD, A. J. 2011b. Forensic validation of the PowerPlex® ESI 16 STR Multiplex and comparison of performance with AmpFlSTR® SGM Plus®. *Int J Leg Med,* 126**,** 345-356.

UNION, C. O. T. E. 2001. *Council resolution of 25 June 2001 on the exchange of DNA analysis results (2001/C 187/01)* [Online]. Official Journal of the European Communities: European Union. [Accessed 19 April 2012].

UNION, C. O. T. E. 2005. *Convention on the stepping up of cross-border cooperation, particularly in combating terrorism, cross-border crime and illegal migration* [Online]. European Union. [Accessed 19 April 2012].

UNION, C. O. T. E. 2008. *Council decision 2008/616/JHA of 23 June 2008on the implementation of Decision 2008/615/JHA on the stepping up of cross-border cooperation,particularly in combating terrorism and cross-border crime* [Online]. Official Journal of the European Union: European Union. [Accessed 19 April 2012].

UNION, C. O. T. E. 2009. *Council Resolution of 30 November 2009 on the exchange of DNA analysis results (2009/C 296/01)* [Online]. Official Journal of the European Union: European Union. [Accessed 19 April 2012].

YURREBASO, I., AJURIAGERRA, J., ALDAY, A., LEZAMA, I., PÉREZ, J., ROMÓN, E., URIARTE, I. & GARCÍA, O. 2011. Allele frequencies and concordance study between the Identifiler and the PowerPlex ESX17 systems in the Basque Country population. *Forensic Sci Int Genet,* 5**,** e79-e80.

ZIEGLE, J. S., SU, Y., CORCORAN, K. P., NIE, L., ERIC MAYRAND, P., HOFF, L. B., MCBRIDE, L. J., KRONICK, M. N. & DIEHL, S. R. 1992. Application of automated DNA sizing technology for genotyping microsatellite loci. *Genomics,* 14**,** 1026-1031.